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**ROLE OF TYPE I INTERFERONS IN THE
TREATMENT OF ENDOCRINE AND NON-
ENDOCRINE CANCERS**

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To Maria Iolanda and my parents

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CHAPTER I:

INTRODUCTION TO TYPE I INTERFERONS



PARTS OF THIS CHAPTER ARE BASED ON:

Caraglia M, **Vitale G**, Marra M, Budillon A, Tagliaferri P, Abbruzzese A. Alpha-interferon and its effects on signaling pathways within the cells. *Current Protein & Peptide Science* 2004;5:475-85.

Caraglia M, **Vitale G**, Marra M, Del Prete S, Lentini A, Budillon A, Beninati S, Abbruzzese A. Translational and post-translational modifications of proteins as a new mechanism of action of alpha-interferon. *Amino Acids* 2004;26:409-17.

I. 1) TYPE I INTERFERONS SUPERFAMILY

In 1957, Alick Isaacs and Jean Lindenmann discovered interferon (IFN). In their experiment they showed that a supernatant, derived from chick chorioallantoic membranes and incubated with inactivated influenza virus, was capable of protecting other membranes from infection by active virus (1). This supernatant contained soluble protein factors capable of inducing a variety of biological functions, called IFNs. The classical function of these proteins is the protection against viral infections. Exposure of cells to viruses and doublestranded RNAs induces the production of IFN- α and IFN- β . However, it emerged soon the ability of IFNs to be involved in other biological processes: cell differentiation, cell growth control, modulation of immune system, and anti-tumor defense (2-5).

There are two major categories of IFNs: type I and type II. IFN- γ is the only type II IFN, whereas the type I IFNs consist of four major classes: IFN- α , IFN- β , IFN- τ and IFN- ω . This thesis will focus on human type I IFNs, in particular the antitumor role of IFN- α and IFN- β in endocrine and non-endocrine cancers. IFN- α comprises a family of different cytokines. There are at least 14 human IFN- α non allelic functional genes found on the short arm of the chromosome 9. They encode for 13 different human IFN- α proteins (IFN- α 1, IFN- α 2, ...) with IFN- α 1 and IFN- α 13 resulting in the identical final protein. All proteins have 166 amino acids except IFN- α 2, which contains 165 subsequent to a deletion at position 44. Some of these subtypes have variant forms (6). The various human IFN- α species have 75-99% amino acids sequence identity and are also highly species-specific with regard to biological properties (6). The human IFN- β gene is present as a single copy also on chromosome 9. The protein is composed of 166 amino acids and there is only approximately 30% amino acid sequence identity between IFN- α and IFN- β (6). A three dimensional, high resolution structure of IFN- α 2a (fig.1) has been generated using heteronuclear NMR spectroscopy (7). In addition, X-ray crystallographic structures have been determined for human IFN- α 2b (fig.2) (8) and natural IFN- β (fig.3) (9). The tertiary structure of type I IFNs contains multiple helices (fig.1-3). However, unlikely the four helices commonly found in other α -helical cytokines, all type I IFNs display a conserved core of five helices (A-E) and one long connection (AB2 loop). The most conserved feature of type I IFNs is the disulfide bond. In IFN- α and IFN- β there are two and one disulfide bonds, respectively. These have an important role in the stabilization of the molecule (7-9). Human IFN- α 2b and IFN- β (fig.2,3) exist in the crystal as a noncovalent dimer, through extensive

interactions mediated by a zinc ion (Zn^{2+}). The IFN- β dimer is different from the IFN- $\alpha 2b$ dimer in that the two molecules are not related by a twofold axis, but by a rotation of about 44° along an axis approximately perpendicular to the plane defined by helices C, B, and D. Thus whereas IFN- $\alpha 2b$ dimerizes with the same contact surface (helix D and AB loop), IFN- β dimerizes with contact surfaces from opposite sides of the molecule. However, the biological significance of these dimers and the different interactions with the receptor are unclear (8,9).

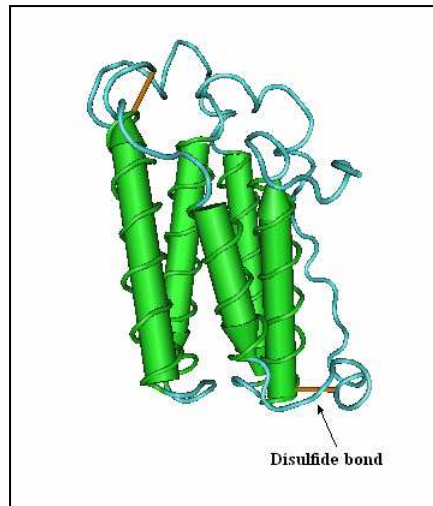


Figure 1: Three dimensional structure of human IFN- $\alpha 2a$ (ref 7)

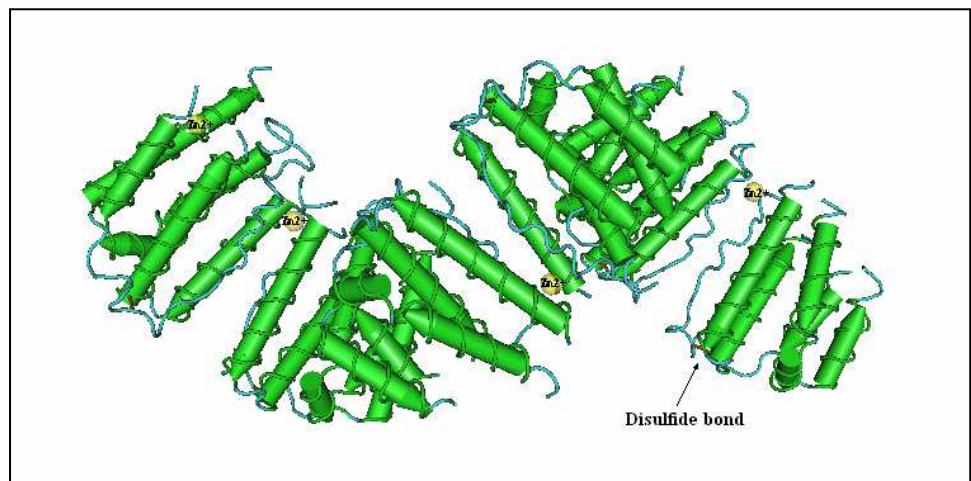
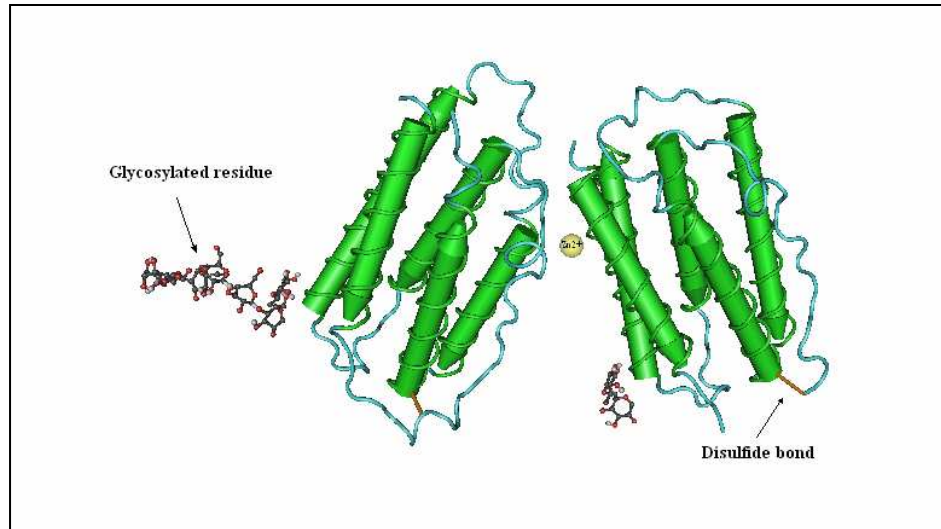


Figure 2: Three dimensional structure of human IFN- $\alpha 2b$. Extensive interactions in the dimer interface are mediated by a zinc ion (Zn^{2+}) (ref.8).

Figure 3: Three dimensional structure of human IFN- β . The modeled portions of the carbohydrates and zinc ion (Zn^{2+}) are also shown (ref.9)



Finally, unlike IFN- α , natural human IFN- β (fig.3) contains a single site, asparagine 80, through which N-linked glycosylation can occur and which has been shown to vary according to the cells in which IFN- β is synthesized (10-11). It is thought that glycosylation plays important roles in facilitating IFN- β secretion from cells, in increasing its solubility and stability, and in its metabolism in vivo (11-16).

At present, only two recombinant alpha IFNs (IFN- α 2a and IFN- α 2b) are available as registered drugs; both appear to have equal activity. Similarly, two types of recombinant human IFN- β are currently in clinical use. IFN- β 1b is produced in *Escherichia coli* bacteria, and as bacteria lack the ability to glycosylate proteins, it is not glycosylated. Furthermore, IFN- β 1b has an amino acid sequence different from the natural human IFN- β ; it has a cysteine-to-serine substitution at position 17 and a deletion of the N-terminal methionine residue. In contrast, IFN- β -1a is produced in mammalian cells, with an amino acid sequence and glycosylation identical to that of natural IFN- β . It has been observed that non-glycosylated IFN- β 1b is much more susceptible to aggregation and is more immunogenic (17). When the protein is not glycosylated, the hydrophobic domain is exposed and the protein will have a tendency to form aggregates as an alternative way to shield this surface, and formation of aggregates is one of the more effective ways to increase the immunogenicity of proteins (18). However, non-glycosylated

IFN- β 1b, although more hydrophobic than glycosylated IFN- β 1a, retains biological activity but with lower specific activity (19,20).

I. 2) TYPE I INTERFERON RECEPTOR

All the cellular effects of type I IFNs appear to be modulated by a common receptor complex, present in low numbers (100-5000 molecules/cell) on the surface of all vertebrate cells examined and composed by two chains (IFNAR-1 and IFNAR-2) that dimerize following the interaction with the ligand (fig.4) (6).

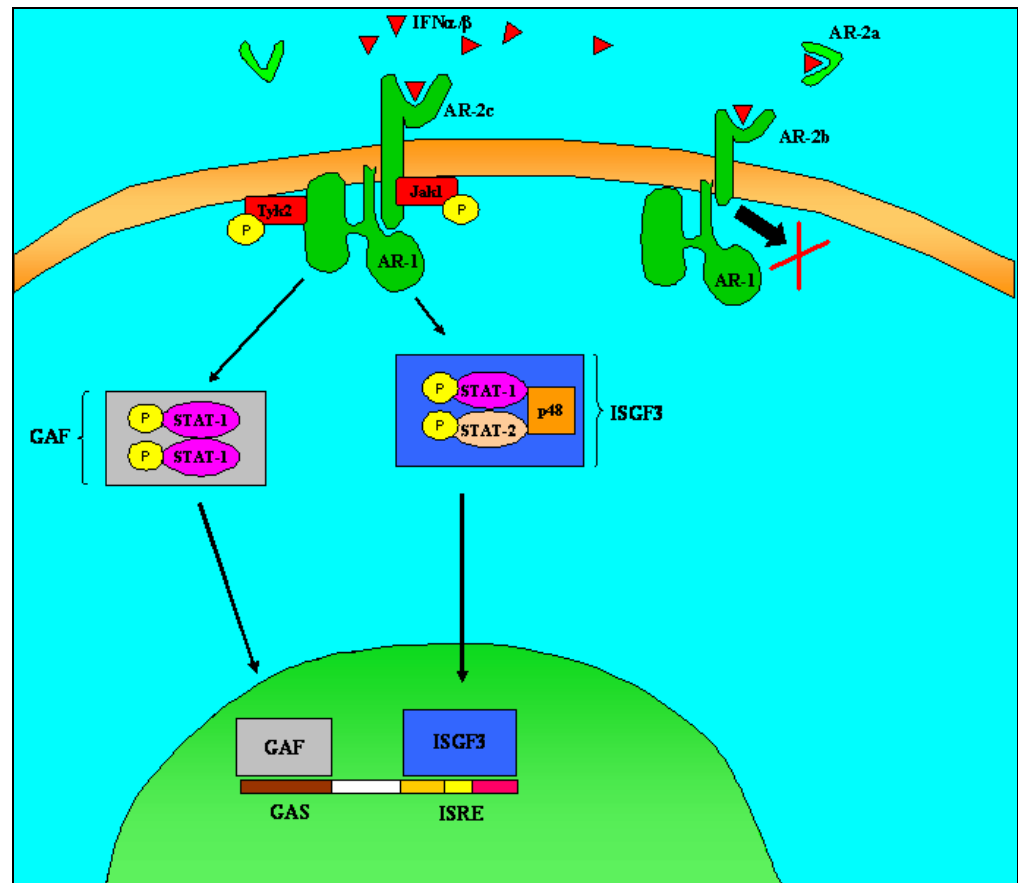


Figure 4: Signal transduction pathways activated by type I IFNs.

IFN α/β , after the interaction with its receptor (AR-1 + AR-2c), activates the tyr kinase Jak-1 and Tyk-2 that are responsible for the activation of the cytoplasmic targets of type I IFNs. The tyr phosphorylation of the targets causes the translocation to the nucleus of STAT-1 and STAT-2 hetero and homodimers, that migrate to the nucleus and bind to DNA mediating the transcription of several genes. Receptors consisting in AR-1 + AR-2b interact with type I IFNs, without transducing signals.

Mature human IFNAR-1, resulting from removal of the peptide sequence leader, is a 530 amino acid residue integral membrane protein. IFNAR-1 is considered the signaling subunit and it exists as the full chain. The IFNAR-2 is the subunit responsible for the interaction with the ligand. Three forms of IFNAR-2 have been isolated, as differentially spliced products of the same gene (fig.4): the soluble (IFNAR-2a), short (IFNAR-2b) and long (IFNAR-2c) form (21-23). The full length receptor chain (IFNAR-2c), comprised of 487 amino acids, together with IFNAR-1 subunit constitute the predominantly active form of the type I IFN receptor complex (fig.4). IFNAR-2c is capable of binding ligand, but with a lower affinity (20-fold less) than the dimeric IFN receptor complex itself (24). Therefore, both receptor chains are required to form a high affinity binding site and initiate signal transduction. The short form (IFNAR-2b), consisting of 303 amino acids of which only 67 residues are in cytoplasmic portion of the molecule, is able to bind type I IFNs, but does not couple to signal transduction because it lacks the signal transducing tail of IFNAR-2c (25). The soluble form (IFNAR-2a), lacking of the transmembrane and cytoplasmic portions, may act as a regulator of free IFNs and, depending on concentration, lead to the neutralization or enhancement of IFN bioactivity (26,27).

After the IFNAR-1 and IFNAR-2c dimerization (fig.4) two intracytoplasmic receptor-associated kinases (Tyk-2, associated with IFNAR-1, and JAK-1, associated with IFNAR-2c) are activated and their activation induces the tyrosine phosphorylation of the receptor itself. In this way the tyrosine phosphorylated sites of the IFN receptor become docking elements for Src homology 2 (SH2) and phosphotyrosyl-binding domain-containing proteins present in the membrane or the cytoplasmic compartment (6). Prominent among these are the Signal Transducer and Activator of Transcription (STATs). These are a family of latent cytoplasmic transcription factors involved in cytokine, hormone, and growth factor signal transduction (28-32). STAT proteins mediate broadly diverse biological processes, including cell growth, differentiation, apoptosis, fetal development, transformation, inflammation, and immune response. Receptor-recruited STATs are phosphorylated on a single tyrosine residue in the carboxyl terminal portion. The modified STATs are released from the cytoplasmic region of the receptor subunits to form homodimers or heterodimers through reciprocal interaction between the phosphotyrosine of one STAT protein and the SH2 domain of another. Following dimerization, STATs rapidly translocate to the nucleus and interact with specific regulatory elements to induce target gene transcription (28-32).

IFN receptor activation classically leads to the phosphorylation and activation of STAT-1 and STAT-2 (fig.4). STAT-1 and STAT-2 form a heterodimer that associates with another cytoplasmatic protein, p48, resulting in the formation of the mature interferon-stimulated gene factor 3 (ISGF3) complex that translocates to the nucleus to initiate gene transcription by binding to interferon-stimulated response elements (ISRE) (33). STAT 1:1 homodimers, STAT 3:3 homodimers, STAT 1:3 heterodimers, STAT 5:5 homodimers, and CrkL:STAT-5 heterodimers are also activated during engagement with type I IFN receptor/IFN- α/β complex. These dimers move to the nucleus where they bind to gamma-activated sequence (GAS) regulatory elements in the promoters of IFN-activated genes (33,34). Thus, signalling specificity via the IFN-activated Jak/STAT pathway is established by the formation of multiple different complexes that activate distinct regulatory elements in the promoters of IFN-regulated genes. This is the most intriguing aspects of the function of type I receptor: its ability to discriminate the various types and subtypes of IFN and to elicit different biological responses. This could be partially explained by the different interactions of IFN- α/β with the common receptor complex. IFN- β induces a real association between IFNAR-1 and IFNAR-2, while IFN- α 1, - α 2, - α 6, - α 7 and - α 8 do not (35). However, in spite of intensive studies, the interactions between IFNs and their receptor complex and the molecular mechanisms underlying the functional biological differences among the IFNs are still largely unknown. More clear are the direct biological effects inducing growth inhibition: stimulation of apoptosis, translational and posttranslational modifications of the proteins and cell cycle arrest. The variability in these direct responses may be influenced by cell type specific differences in STAT complement, availability of coactivators and oncogenic alterations present in the target cell (36).

I. 3) INDUCTION OF APOPTOSIS

Apoptosis plays an important role in the control of many normal physiological processes, such as embryonic development, immune regulation and maintenance of tissue homeostasis (37). Decreased sensitivity to apoptotic stimuli is a trait commonly shared by cancer cells. This feature provides the tumour cells with a survival advantage, facilitating the out-growth of malignant clones and may also explain a variable susceptibility to various anti-cancer drugs (38,39). Type I IFNs induce apoptosis in different ways (fig.5), influencing the death receptor (extrinsic pathway) and the

mitochondrial pathway; in this way, it could play a role in the antitumor activity and clearing of virus-infected cells (40, 41).

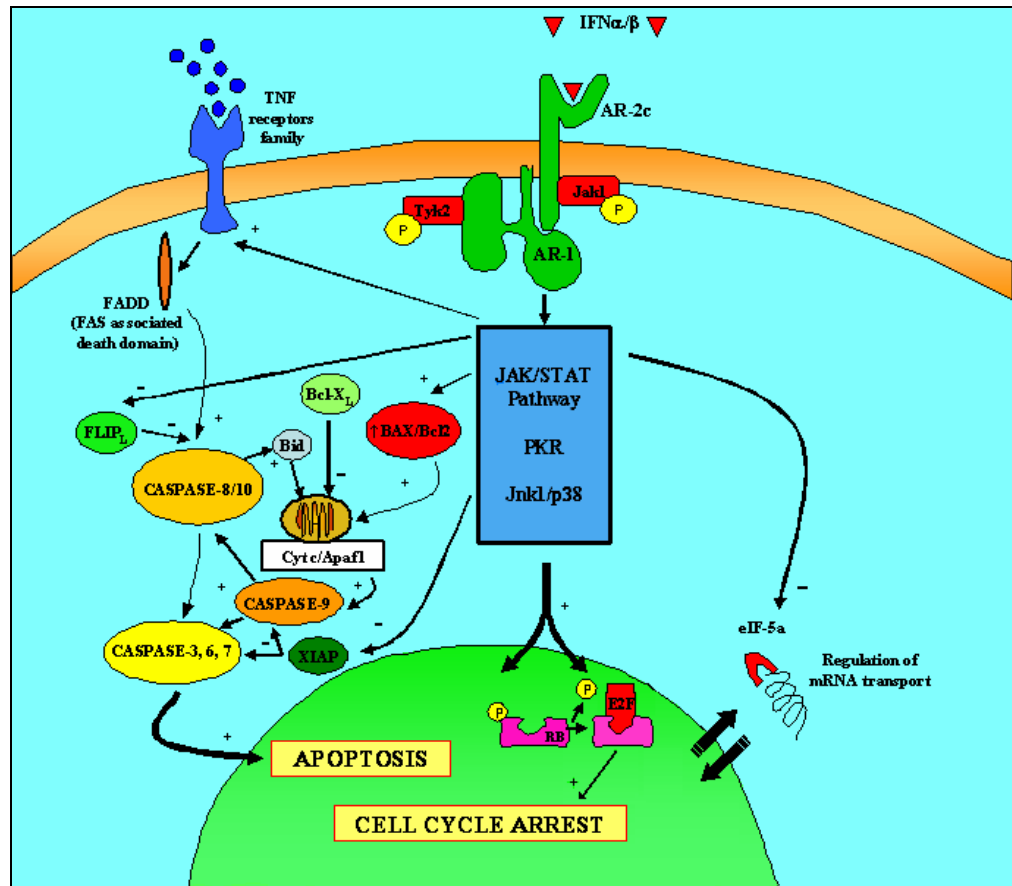


Figure 5: The regulation of apoptosis and cell cycle by the type I IFNs. Activation of STAT pathways by the type I IFN/tyrosine kinases (Jak/Tyk-2) complex leads to induction of proapoptotic machinery through caspase cascade activation depending upon Fas system up-regulation, Jnk1/p38 stimulation or still unidentified mechanisms; inhibition of cell cycle progression via p21/p27 increased expression, suppression in the phosphorylation of pRb, p130 and p107.

The Extrinsic Pathway

Apoptosis can be mediated by the activation of death receptors belonging to the tumor-necrosis factor (TNF) receptor family (fig.5). Members of the TNF receptor family share a common internal domain, the so-called death domain, and are activated by several ligands, including TNF- α , Fas ligand and TRAIL (TNF-related apoptosis-inducing ligand). The interaction between receptor and ligand recruits FADD (Fas-associated death domain protein) and procaspase-8 to the death domain forming the DISC (death-inducing signaling complex). At the DISC, cleavage of procaspase-8 yields the active form of this protease. In type I cells, the amount of activated caspase-8 is sufficient to

initiate apoptosis via direct activation of the central effector caspase-3 (40,41).

In myeloma, as well as in glioma cell lines, long term treatment with IFN has been suggested to sensitize the cells to Fas-induced apoptosis (42,43). Moreover, the Fas ligand/receptor system may mediate effects of IFN- α 2 in basal cell carcinoma (44). In fact, after injection of IFN- α 2 into basal cell carcinomas, Fas receptor and apoptosis were induced, and tumours regressed. However, Chawla-Sarkar et al. have recently demonstrated that IFN- β is a stronger Fas system and apoptosis inducer than IFN- α in melanoma cells (45). Similar data were obtained by Sanceau et al. in sarcoma cell lines in which IFN- β induces p38 MAPK-mediated Ser 727 STAT-1 phosphorylation and apoptosis more efficiently than IFN- α (46). Studies on T cells have shown a downregulation of FLICE-like inhibitory protein (FLIP) and inhibitor apoptosis proteins (IAP), both inhibitor regulators of apoptosis process, following treatment with IFN- β (fig.5) (41). Despite these biological differences the molecular basis of the diversity between IFN- α and IFN- β in the induction of apoptotic events is still unknown.

The Mitochondrial Pathway

In type II cells, the signal enhancing-effect of mitochondria is needed to induce the full apoptotic process. The detailed molecular background to IFN-induced apoptosis remains unclear, but it was demonstrated that it also involves an ordered activation of mitochondrial pathway, as shown by the release of cytochrome c (cyt c) and the association with apoptosis-activating factor 1 (Apaf-1) and procaspase-9 to form the apoptosome complex. This leads to the activation of caspase-9, which induces in turn executioner caspases (caspases-3, -6, -7) (fig.5) (47).

Moreover, Thyrrell et al. have demonstrated that IFN- α -induced apoptosis is not inhibited by antagonistic antibodies to the Fas-receptor and, thus, it is a Fas-independent effect (32).

In a similar experimental model, Panaretakis et al. have showed that IFN- α -induced apoptosis occurs together with the activation of the pro-apoptotic Bcl-2 related proteins Bak and Bax (48). However, Bak activation occurred early in the apoptotic response, prior to the cytochrome c release and loss of mitochondrial membrane potential, whereas Bax activation followed these events (49). The same authors showed a transient initial increase of two inhibitors of apoptosis (Bcl-xL and Mcl-1), that could explain the late onset of the apoptosis induced by IFN- α (49). Taken together these results suggest a mitochondrial involvement in the apoptosis triggered by type I IFNs (50).

The Stress Kinase Cascade Involvement

There are indications that other pathways affecting the regulation of apoptosis can also be modulated by type I IFNs. In eukaryotic cells enzymatic isoforms of MAPK, such as Jun kinase-1 (JNK1) and p38 kinase, mediate antiproliferative stimuli and apoptosis. They have large sequence homology, but are functionally different from proliferative pathway-associated Erk-1/2. In fact, JNK1 and p38 kinase are part of enzymatic cascades activated by antiproliferative agents such as ionizing and ultraviolet rays and cytokines.

In this regard, there are several evidences that type I IFNs activate members of the MAPK family, including Erk (51), the p38 MAPK (that belongs to the stress-activated kinases) and JNK-1 (52-54). In addition, p38 seems to be essential for the transcription of IFN-dependent genes, independently of activation of STAT protein (52-55).

It was, moreover, found that p38 MAPK pathway is engaged in type I IFN signalling in primary human hematopoietic progenitors and its function is required for the generation of the suppressive effects of IFNs on normal hematopoiesis (56). Moreover, p38 MAPK, is involved in the generation of the antileukemic effects of IFN- α in break cluster region (BCR)-ABL-expressing cells of acute myeloid leukemia (57).

Another gene involved in the IFN-mediated apoptosis is the tumour suppressor gene p53. Takaoka et al. (58) have recently shown that transcription of the p53 gene is induced by IFN- α/β , accompanied by an increase in p53 protein level. IFN α/β signalling itself does not activate p53; rather, it contributes to boosting p53 responses to stress signals. In these experimental conditions p53 gene induction by IFN- α/β contributes to tumour suppression, is activated in virally infected cells to evoke an apoptotic response and is critical for antiviral defence of the host (58).

Finally, the role of NF- κ B in the apoptosis induced by IFN- α is controversial. In fact, it has been demonstrated that IFN- α can activate NF- κ B through STAT-3 and via phosphatidylinositol 3 kinase (PI3K) and Akt activation in lymphoma cells and promotes survival of human primary B-lymphocytes via PI3K (59). Other studies demonstrate that IFN- α sensitizes human hepatoma cells to TRAIL-induced apoptosis through DR5 upregulation and NF- κ B inactivation or suppresses the antiapoptotic effect of NF- κ B and sensitizes renal cell carcinoma cells *in vitro* to chemotherapeutic drugs (60,61).

For a summary see Table 1.

Table 1. Modes by which type I IFNs induce apoptosis

General Mechanism	Molecular target	Ref
<i>Death receptor and mitochondrial involvement</i>	Fas Receptor/Ligand	42-45
	Cyt-c release and loss of mitochondrial membrane potential	47
	Fas-independent Caspase 9 activation	32
	Bak and Bax activation	48
<i>Stress kinase cascade</i>	P38 kinase and consequent activation of MapK α K-2	52-54
	JNK-1 and P38 kinase	55
	P53	58
	STAT-3->NF- κ B->Akt	59
	-Sensitization to TRAIL	60-61
	- DR5 increase->NF- κ B inactivation	

I. 4) TRANSLATIONAL AND POST-TRANSLATIONAL MODIFICATIONS OF PROTEINS

Protein kinase dependent from double-stranded RNA (PKR)

IFNs regulate at the transcriptional level more than 200 gene products that determine their responses. The protein kinase dependent from double-stranded RNA (PKR), involved in protein synthesis regulation, is one of gene stimulated by type I IFNs. Human PKR is a serine-threonine kinase of 551 amino acid residues, implicated in the promotion of cell death mediated by double-stranded RNA. Once activated, PKR phosphorylates exogenous substrates. The best characterized substrate of PKR is the small subunit of the eukaryotic initiation factor 2 (eIF2). PKR inhibits translational initiation by phosphorylating eIF2 on serine (62). Additionally, PKR also regulates the action of several transcription factors such as NF- κ B, IRF-1, p53, STAT-1 and NF-90. Modulation of all of these different targets allows PKR to control diverse cellular processes, such as cell growth (63), differentiation (64) and to exert antitumour activity (65), by induction of cell cycle arrest and, mainly, of apoptosis. In fact, PKR activation regulates translational and transcriptional pathways (eIF-2 α and NF- κ B-dependent) resulting in the specific expression of selected proteins (Fas, p53, Bax and others) that trigger cell death by engaging with the caspase pathway (66).

The eukaryotic initiation factor-5A of protein synthesis (eIF-5A)

The eIF5A belongs to a family of translational factors that are involved in the regulation of cell growth. eIF5A is peculiar because its activity is modulated

by a series of post-translational modifications that culminates in the formation of the unusual amino acid hypusine. Hypusine [N^{ϵ} -(4-amino-2-hydroxybutyl)lysine] is formed by the transfer of the butylamine portion from spermidine to the ϵ -amino group of a specific lysine residue of eIF-5A precursor and by the subsequent hydroxylation at carbon 2 of the incoming 4-aminobutylmoiety (67). eIF-5A probably acts in the final stage of the initiation phase of protein synthesis by promoting the formation of the first peptide bond (68). Hypusine plays a key role in the regulation of eIF-5A function. In fact, only the hypusine-containing eIF-5A form is active and, consequently, the dosage of intracellular hypusine content measures also the activity of eIF-5A, since hypusine is contained only in this factor (69). The correlation between hypusine, eIF-5A activity, and cell proliferation suggests that activated eIF-5A might play a role in cell growth and differentiation (70). The cell proliferation regulatory properties of eIF-5A could be correlated by its reported mRNA chaperon functions since eIF-5A is involved in the transport of mRNAs from the nucleus to the cytoplasm (fig.5) (71). It has been also proposed that these mRNAs could encode for proteins involved in the regulation of cell proliferation (72).

Caraglia et al. reported that IFN- α induces growth inhibition and reduction of the activity of eIF-5A in human epidermoid cancer KB cells (fig.5) (73). The activity of eIF-5A was evaluated through the determination of hypusine levels since this amino acid is essential for the function of this translational factor that is involved in the regulation of cell proliferation and transformation. IFN- α is able to induce a strong inhibition of eIF-5A activity (fig.5) since a reduction of hypusine synthesis was recorded with a parallel increase of eIF-5A protein expression. This finding suggests a further reduction of the active fraction of eIF-5A (hypusine-containing eIF-5A:total eIF-5A ratio) (73). Moreover IFN- α induces cell growth inhibition and apoptosis in human epidermoid cancer cells and these effects are antagonized by EGF. On the other hand, when EGF antagonized the apoptosis induced by IFN- α , a restoration of hypusine synthesis caused by the cytokine and an increase of extracellular signal regulated kinase (Erk) activity are recorded in cancer cells (73). All these data support the hypothesis of an involvement of eIF-5A, another protein synthesis regulator, in the apoptosis induced by IFN- α , at least in human epithelial cells.

The modulation of protein degradation

The proteasome is a multisubunit enzyme complex that plays a central role in the regulation of proteins that control cell-cycle progression and apoptosis, and has therefore become an important target for anticancer therapy. In fact,

the expression of proteins essential for the regulation of cell growth and survival can be also controlled at post-transcriptional and post-translational levels, the latter through the regulation of protein degradation. Before a protein is degraded, it is first flagged for destruction by the ubiquitin conjugation system, which ultimately results in the attachment of a polyubiquitin chain on the target protein. The proteasome's 19S regulatory cap binds the polyubiquitin chain, denatures the protein, and feeds the protein into the proteasome's proteolytic core. The proteolytic core is composed of 2 inner beta rings and 2 outer alpha rings. The 2 beta rings each contain 3 proteolytic sites named for their trypsin-like, post-glutamyl peptide hydrolase-like (i.e., caspase-like), or chymotrypsin-like activity. Inhibition of the proteasome generally results in cell-cycle arrest and apoptosis (74).

It has been demonstrated that type I IFNs can regulate this complex system. Since 1996 it has been demonstrated that type I IFNs can regulate the expression of a 16-kDa protein that is produced by the bovine endometrium during early pregnancy and that shares epitopes with ubiquitin and ubiquitin cross-reacting proteins (75). In addition, IFN- α induces the expression of an ubiquitin cross-reactive protein (ISG15) and two ubiquitin-conjugating enzymes (UbcH5 and UbcH8) in T cells. A molecular target of these enzymes is the inhibitor of κ B (IkB) protein, a cytoplasmic protein that binds and inactivates NF- κ B. IkB degradation activates Rel/NF- κ B that triggers the transcription of genes involved in the apoptotic/antiapoptotic process. (76)

More recently, it was demonstrated that administration of interleukin-1 β (IL-1 β) in vivo attenuates IFN- α -induced STAT-1 tyrosine phosphorylation in the liver but not in the spleen. The inhibitory action of IL-1 β in vivo is not affected by depleting hepatic Kupffer cells, suggesting that IL-1 β may directly target IFN- α signalling in hepatocytes. Indeed, pretreatment of human hepatocellular carcinoma HepG2 cells with IL-1 β suppresses IFN- α -induced antiviral activity and protein MxA mRNA expression. Furthermore, IL-1 β attenuated IFN- α -induced STAT-1 binding and tyrosine phosphorylation without affecting the level of STAT-1 protein. This inhibitory effect can be reversed by pretreatment with either proteasome inhibitors or transfection of dominant negative NF- κ B inducing kinase mutants. Taken together, these findings suggest that IL-1 β attenuates IFN- α -induced STAT-1 activation by a proteasome-dependent mechanism (77).

In human epidermoid lung cancer cells IFN- α induces apoptosis through, at least in part, the increase of the expression and activity of tTGase, a ubiquitous member of the transglutaminase enzyme family, that has been reported to play a role in apoptosis (78). The increase of the expression of

tTGase was not due to the induction of its transcription, but to a decrease of its degradation via a proteasome-dependent pathway.

Therefore, type I IFNs could modulate apoptosis through the regulation of the degradation of intracellular proteins involved in the triggering of apoptotic process. However, not only the target of the type I IFN-dependent signalling but also the components of the pathway activated by the cytokine itself can be subjected to regulation via proteasome-dependent degradation. In fact, Mumps virus, a common infectious agent of humans, causing parotitis, meningitis, encephalitis, and orchitis, induces degradation of STAT-3 mediated by the ubiquitination and subsequent proteasome-dependent degradation (79). The latter could be a mechanism by which viruses protect themselves by the anti-viral action of type I IFNs. Finally, the suppressors of cytokine signalling (SOCS) are a family of physiological regulators of cytokine responses, including the type I IFNs. One of the modes used by SOCS to turn off the IFN- α -dependent signalling is the delivery of the transductional components to the degradative proteosomal machinery (80). For a summary see Table 2.

Table 2. Correlations between the protein degradation and the type I IFNs-dependent pathways

Proteasome component	Target	Source	Biological Effect	Ref
16kD protein similar to ubiquitin and ubiquitin cross-reacting protein	Unknown	Bovine endometrium during pregnancy	Unknown	75
<ul style="list-style-type: none"> Ubiquitin cross-reactive protein Ubiquitinating enzymes (UbcH5 and UbcH8) 	Unknown	T cells	Unknown	76
Unknown	STAT-1	Hepatocellular carcinoma cells	Attenuation of IFN- α activity by IL1 β	77
Unknown	TTG	Epidermoid cancer cells	Mediates apoptosis induced by IFN- α	78
Unknown	STAT-3	Epithelial cells	Attenuation of IFN- α activity by Mumps virus	79
Unknown	IFN-AR, JAK-1, Tyk-2, STATs	Epithelial cells	Attenuation of IFN- α activity by the inactivator SOCS	80

I. 5) CELL CYCLE ARREST

The arrest of cell cycle is another antitumor mechanism described in several cancers after treatment with type I IFNs. IFN- α and IFN- β can affect all phases of the mitotic cell cycle (81). Infact, type I IFNs have been described to induce a G₀-G₁ arrest, to prolong S-phase transition or to block cells in G₂/M.

Temporal order and continued passage through cell cycle is dependent on the sequential formation and activation of series of serine/threonine protein kinases. These protein complexes consist of a regulatory subunit, termed cyclin, and a catalytic subunit, the cyclin-dependent kinases (cdks) (fig.6). From G₁, following mitosis, cells may also enter a stable “resting state” called quiescence, or G₀. As cells enter the cycle following mitosis (or alternatively from quiescence), the D-type cyclins (D1, D2 and D3) and cyclin E are synthesized sequentially, leading to the formation of cyclin D-cdk4/6 and cyclin E-cdk2 complexes. Other cdk-complexes, such as cyclin A-cdk2, operate in S and G₂, whilst cyclin B-cdk1 functions in G₂/M phase. To ensure fine-tuning in the regulation of proliferation, the activity of these complexes is regulated at multiple levels, including the synthesis and destruction of the regulatory cyclin, and by phosphorylation and dephosphorylation on specific residues of the kinase subunit by the enzymes CAK and Cdc25A, respectively. In addition, a new level of regulation of these complexes has been elucidated by the identification of two families of low molecular weight cdk inhibitors. This inhibitors include the Ink4 family (p15, p16, p18 and p19 proteins), which binds specifically to cdk4 and cdk6, thereby interfering with cyclin D-cdk interactions; and Cip/Kip family (p21, p27 and p57 proteins), inhibiting the kinase activity of cyclin E-cdk2, cyclin A-cdk2 and cyclin D-cdk4/6 complexes (82).

Many proteins have been suggested as substrates for the different cyclin/cdk complexes. A main substrate of cyclin D-cdk4/6 is the pRb protein that influences the transcription of genes necessary for G₁ progression through binding and regulating the activity of members of the E2F family of transcription factors as well as histone deacetylases (HDAC:s). The phosphorylation of pRb by cyclin D-cdk4/6 induces a conformational change, which disrupts its binding to HDAC and causes de-repression of some genes with E2F sites in their promoters. Further phosphorylation of pRb, and the two other pocket proteins p107 and p130 by cyclin E-cdk2 leads to release of E2F and transactivation of E2F-responsive genes required for S phase progression, such as DNA polymerase alpha, dihydrofolate reductase, and c-myc. Therefore, the phosphorylation of the pRb protein by cyclin/cdk complexes is a rate limiting step in G₁ progression (83).

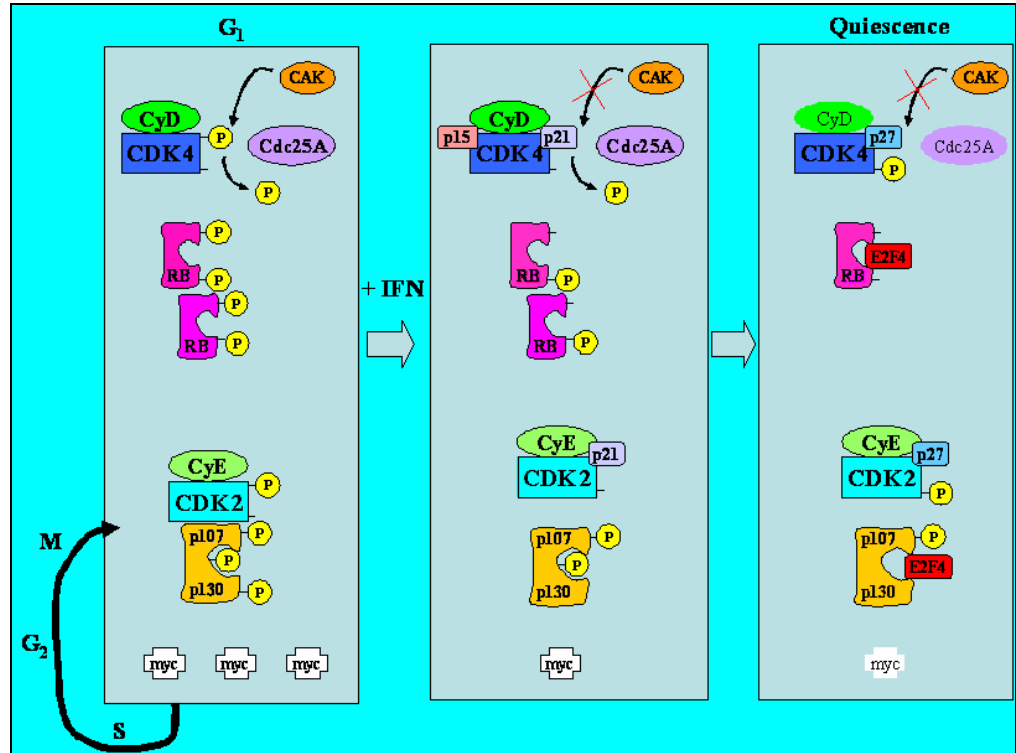


Figure 6: The proposed model for type-I IFNs induced G_0 -like arrest. Addition of type I IFN to exponentially growing cells can cause a G_0 -like arrest. This is associated with a down-regulation of Cdk4 and Cdk2 activity, presumably leading to activation of the pocket proteins. This effect is thought to be brought about through a primary response involving the transcriptional induction of the Cdk inhibitors p15 and p21. This is followed by a secondary response involving a post-translationally regulated increase in p27 levels, down-regulation of cyclins D and A, as well as down-regulation of the Cdk-activating phosphatase Cdc25A. The binding of Cip/Kip inhibitors to the G1 Cdk complexes also leads to a blockage of their phosphorylation by the CAK enzyme. In parallel with the above-mentioned processes the levels of c-myc are rapidly downregulated (*modified from Sangfelt O. et al., ref. 82*).

IFN- α induced a cell accumulation in a G_0 -like state and a cell cycle arrest in G_1 phase in Daudi cells and in U-266 cells, respectively (83). These effects seem to be modulated by the suppression in the phosphorylation of pRb, p130 and p107 (82,83).

Since pocket protein phosphorylation is regulated by the cdk-complexes, several groups have addressed the issue of whether IFN acts by down-regulating cdk activity. Indeed, type I IFNs have been shown to strongly repress the activity of the cdk2 –4 and 6-complexes in a number of different cell types. There are several possible explanations for this effect, as there are many ways of regulating cdk activity. First, IFN could act by decreasing the levels of the cdk or their regulatory cyclin subunits. Second, it could increase the expression of one or several cdk-inhibitory proteins, or, thirdly, influence the phosphorylation status of the cdk by affecting CAK activity or expression of Cdc25A (fig.6) (82,83). Experimental data have shown that, indeed, IFN

influences cdk activity through all of these mechanisms (82). In fact, long incubation with IFN- α decreases cyclin D3 and cyclin A expression and activity in the Burkitt lymphoma cell line Daudi (82). The induction of several cdk-inhibitory proteins (p21, p15, p27) in parallel to the decrease in the activity of G1 cdks has been detected during IFN- α treatment. In particular, the increase in p27 levels induced a dissociation of cyclin E/Cdk2-p130 or p107 complexes to yield cyclin E/Cdk2-p27 complexes (83, 84). As mentioned above, full cdk activity is only acquired if the kinase subunit is dephosphorylated at certain residues and phosphorylated on others. This requires both the phosphatase CDC25a and the cdk-complex CAK. Type I IFNs seem also to down-regulate the expression of Cdc25A and to block the activity of CAK. The overall reduction in activities of cyclins and cdk, resulted in decreased pRb phosphorylation, thereby preventing cell cycle progression into S phase (85).

Another involved pathway could be the activation of the double-stranded RNA-activated protein kinase (p68 PKR), inducing cell cycle arrest in G₁ through the proto-oncogene *c-myc* suppression (86,87).

Other studies revealed S-phase accumulation in response to IFN- α and IFN- β , as a failure to complete DNA replication. In hepatocellular carcinoma, where IFN- α induced an accumulation in S-phase, the cell cycle-dependent induction of cyclin A and cyclin B was impaired, resulting in reduced activity of cdk2 and cdc2 kinases. Because cyclin A expression is absolutely required for passage through S phase, while cyclin B is synthesized to function as a regulatory subunit in cdc2 kinase complexes as cells progress from S- into G₂/M, the decrease of cellular cyclin A and B content represents a likely cause for IFN- α -dependent growth inhibition (88). Similar results were observed by Detjen et al. in neuroendocrine tumor cells (89). Qin et al. reported the loss or inactivation of the normal G₁ checkpoint conferred by the Rb protein as a mechanism of IFN- α and IFN- β -mediated accumulation of the cells in the S phase. The increase appeared to be due to a continued S phase entry and subsequently a failure of S phase cells to transit efficiently into G₂ and M phases (90).

Finally, few papers reported a blockage at the G₂/M phase after type I IFNs treatment, but the involved mechanisms are not completely clear (91,92).

I. 6) THE AIMS OF THE PRESENT THESIS:

The current thesis has been designed with the following aims:

1. To compare the anti-tumor effects of IFN- α and IFN- β and the involved mechanisms in: pancreatic carcinoid (BON cell line), human pancreatic adenocarcinoma (BxPC-3, MiaPaCa-2 and Panc-1 cell lines) and adrenocortical cancer (H295 and SW-13 cell lines).
2. To evaluate the mechanisms of tumor cell resistance to IFN- α , related to the up-regulation of epidermal growth factor receptor (EGF-R) in epidermoid cancer cells (KB cell line).

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CHAPTER II:

INTERFERON-BETA IS A HIGHLY POTENT INHIBITOR OF GASTROENTEROPANCREATIC NEUROENDOCRINE TUMOR CELL GROWTH IN VITRO



Vitale G, de Herder WW, van Koetsveld PM, Waaijers M, Schoordijk W, Croze E, Colao A, Lamberts SWJ, Hofland LJ. *Cancer Research* 2006; January (in Press).

Abstract

Interferon (IFN)- α controls hormone secretion and symptoms in human gastroenteropancreatic neuroendocrine tumors (GEP-NETs), but it rarely induces a measurable tumor size reduction. The effect of other type I IFNs, i.e. IFN- β , has not been evaluated. We compared the antitumor effects of IFN- α and IFN- β in BON cells, a functioning human GEP-NET cell line. As determined by quantitative RT-PCR analysis and immunocytochemistry, BON cells expressed the active type I IFN receptor mRNA and protein (IFNAR-1 and IFNAR-2c subunits). After 3 and 6 days of treatment, IFN- β significantly inhibited BON cell growth in a time- and dose-dependent manner. IC₅₀ and maximal inhibitory effect on day 6 were 8 IU/ml and 98%, respectively. In contrast, the effect of IFN- α resulted significantly in a less potent effect (IC₅₀: 44 IU/ml, maximal inhibition: 26%). IFN- α induced only cell cycle arrest, with an accumulation of the cells in S phase. IFN- β , apart from a more potent delay in S-G2/M phase transit of the cell cycle, also induced a strong stimulation of apoptosis, evaluated by flow cytometry (annexin V and 7-AAD) and measurement of the DNA fragmentation. Besides, only IFN- β severely suppressed chromogranin A levels in the medium from BON cells after 6 days of treatment. In conclusion, IFN- β is much more potent, compared to IFN- α , in its inhibitory effect on GEP-NET cell proliferation in vitro through the induction of apoptosis and cell cycle arrest. Further studies are required to establish whether IFN- β has comparable potent tumor growth inhibitory effects in vivo.

Introduction

Gastroenteropancreatic-neuroendocrine tumors (GEP-NETs) are a heterogeneous category of tumors and represent the largest group of all NETs (1). GEP-NETs frequently synthesize several biologically active substances, (bioamines and neuropeptides), leading to disease characterized by diarrhea, abdominal cramps and pain, cardiac valve disease, bronchoconstriction, flushing, telangiectasias and pellagra-like dermatitis, often debilitating for patients (1-3). In the liver, monoamine oxidases can detoxify the amines released by the tumor and prevent the occurrence of the tumor-associated hypersecretion syndrome. However, the presence of hepatic and occasionally large retroperitoneal and ovarian metastases, where secreted products by-pass the liver detoxification, is commonly associated to this syndrome (4).

In addition to the impaired quality of life, patients with advanced GEP-NETs have a dismal prognosis with a 5-year survival of about 20-30% (5). In these cases there are relatively few therapeutic options, most of them are palliative. Surgery is indicated only for conservative resections of the intestine, mesenteric tumors and fibrotic areas, in order to improve symptoms and quality of life (6). Hepatic resection in selected patients with metastatic functioning GEP-NETs is safe, provides temporary relief of symptoms, and may prolong survival (6,7). As alternative to hepatic resection in patients with liver metastases, local ablative procedures may also be considered including chemoembolization, laser and radiofrequency ablation, as palliative support to control symptoms of hypersecretion syndrome (6). The use of chemotherapeutic regimens is limited to fast growing or undifferentiated tumors (8). Few studies suggest the use of targeted radiotherapy with somatostatin analogues coupled to β - or Auger electron emitters in the management of GEP-NETs, but its definitive role has yet to be defined (9-11). The most common medical treatment in advanced GEP-NETs includes the use of somatostatin analogs and interferon (IFN)- α , alone or in combination. Somatostatin analogs are used in the treatment of clinical symptoms, but tachyphylaxis is frequently recorded and these drugs are unable to control tumor progression (12-13). IFN- α induces a 42% biochemical and 14% tumor response in carcinoid, and a 51% biochemical and 12% tumor response in neuroendocrine pancreatic tumors (14-15). However, also after the treatment with IFN- α a measurable tumor reduction is only reported occasionally (16). Therefore, therapeutic options to inhibit the growth of metastatic GEP-NET tumors are still unsatisfactory, and many patients become refractory to the conventional palliative therapy. For all these reasons, novel treatment strategies appear mandatory. Few authors reported that IFN- β has greater antitumor effects than IFN- α on melanoma, squamous carcinoma, glioma,

breast and hepatocellular cancer (17-21). On these bases, IFN- β appears to be of potential interest in the treatment of tumors.

To further explore the possibilities of new medical therapies in GEP-NETs, we compared in this study the role and mechanism of action of IFN- α and IFN- β in the regulation of cell growth in BON cells, a functioning human NET cell line, derived from a lymph node metastasis of a pancreatic carcinoid.

Materials and methods

Cell lines and culture conditions

Human pancreatic carcinoid BON cells were obtained from Dr Townsend (The University of Texas Medical Branch, Galveston, Texas, USA). The cells were cultured in a humidified incubator containing 5% CO₂ at 37°C. The culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12K medium, supplemented with 10% FCS, penicillin (1x10⁵ U/l), fungizone (0.5 mg/l) and l-glutamine (2 mmol/l). Periodically, cells were confirmed as *Mycoplasma*-free. Cells were harvested with trypsin EDTA 10% and resuspended in medium. Before plating, cells were counted microscopically using a standard hemocytometer. Trypan Blue staining was used to assess cell viability, which always exceeded 95%. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

Drugs and Reagents

Human recombinant IFN- α -2b (Roferon-A) was obtained from Roche (Mijdrecht, The Netherlands). Human recombinant IFN- β -1a was obtained from two preparations: Serono Inc. (Rebif, Rockland, MA) and GIBCO Brl (Paisley, UK). All compounds were stored at -20°C, and the stock solution was constituted in distilled water according to the manufacturer instructions. Anti-human IFN- β neutralizing antibody was purchased from Sigma-Aldrich (St. Louis, MA).

Quantitative RT-PCR

The expression of type I IFN receptors (IFNAR-1, IFNAR-2 total, the short form IFNAR-2b, and the long form IFNAR-2c) and housekeeping gene hypoxanthine-phosphoribosyl-transferase (HPRT) mRNA was evaluated by quantitative RT-PCR in BON cells. Poly A⁺ mRNA was isolated using Dynabeads Oligo (dT)₂₅ (DynaL AS, Oslo, Norway) from cell pellets containing approximately 5x10⁵ cells. The cells were lysed for 2 minutes on ice in a buffer containing 100 mM Tris-HCl (pH 8), 500 mM LiCl, 10 mM EDTA (pH 8), 1% LiDS, 5 mM DTT and 5 U/100 ml of RNase inhibitor (HT Biotechnology Ltd. Cambridge, UK). To the lysate 30 μ l prewashed Dynabeads Oligo (dT)₂₅ were added, and the mixture was incubated for 10 minute on ice. Thereafter, the beads were collected with a magnet, washed three times with 10 mM Tris-HCl (pH 8), 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS, and two times with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 2 x 20 μ l of H₂O for 2 min at 65°C. Complementary DNA (cDNA) was synthesized using the poly A⁺ mRNA in a Tris-buffer (50 mM Tris-HCl, pH 8.3; 100 mM KCl; 4 mM DTT; 10mM MgCl₂) together with 1mM of each deoxynucleotide triphosphate, 10 U RNase inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 40 μ l. This mixture was incubated for 1 h at 42°C. One tenth of the cDNA library was used for quantification of IFN receptors and HPRT mRNA levels. A quantitative PCR was performed by AmpliTaq Gold® DNA Polymerase and the ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Groningen, The Netherlands) for real-time amplifications, according to manufacturer's protocol. The assay was composed by 15 μ l TaqMan® Universal PCR Master Mix (Applied Biosystems, Capelle aan de IJssel, The Netherlands), forward primer (500 nmol HPRT and IFNAR-2c; 300 nmol IFNAR-1, IFNAR-2 total and IFNAR-2b), reverse primer (500 nmol HPRT and IFNAR-2c; 300 nmol IFNAR-1, IFNAR-2total, and IFNAR-2b), probe (100 nmol HPRT,

IFNAR-1 and IFNAR-2total; 200 nmol IFNAR-2b and IFNAR-2c) and 10 µl cDNA template, in a total reaction volume of 25 µl. PCR amplification started with a first step for 2 min at 50°, followed by an initial heating at 95°C for 10 min, samples were subjected to 40 cycles of denaturation at 95°C for 15 sec and annealing for 1 min at 60°C. The primer and probe sequences were purchased from Biosource (Nivelles, Belgium) and are indicated in Table 1.

Table 1: Primers and probes used for the quantitative RT-PCR.

IFNAR-1	
Forward	5'-CCCAGTGTGTCTTTCCTCAAA-3'
Reverse	5'-AAGACTGGAGGAAGTAGGAAAGC-3'
Probe	5'-FAM-TCCGCGTACAAGCATCTGATGGA-TAMRA-3'
IFNAR-2 (total form)	
Forward	5'-AGTCAGAGGGAATTGTGAAGAAGCA-3'
Reverse	5'-TTTGGAATTAAGTGTCAATGATATAGGTG-3'
Probe	5'-FAM-AAACCCGAAATAAAGGAAACATGAGTGGAAATT-TAMRA-3'
IFNAR-2b (short form)	
Forward	5'-GCTTAAGAAATAGCCTCCCCAAA-3'
Reverse	5'-CTGTGAATAGCCACTGCATTCC-3'
Probe	5'-FAM-TCTTGAGGCAAGGTCTCGCTAAGGGC-TAMRA-3'
IFNAR-2c (long form)	
Forward	5'-TGACAAGCACCATAGTGACACTGA-3'
Reverse	5'-TAGGAAATGGCCAGGCTAAAAA-3'
Probe	5'-FAM-TGGATTGGTTATATATGCTTAAGAAATAGCCTCCCCA-TAMRA-3'
HPRT	
Forward	5'-TGCTTTCCTTGGTCAGGCAGTAT-3'
Reverse	5'-TCAAATCCAACAAAGTCTGGCTTATATC-3'
Probe	5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'

The detection of HPRT mRNA was used for normalization of IFN receptor mRNA levels. Expression of IFNAR-2a mRNA, the soluble form of IFNAR-2 subunit, was determined indirectly by subtracting IFNAR-2b and IFNAR-2c from IFNAR-2 total. Several controls were included in the RT-PCR experiments. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of HPRT and type I IFN receptors human cDNA was amplified in parallel with the cDNA samples.

Immunocytochemistry

Cytospin preparations of BON cells were fixated with acetone and subsequently incubated for 30 minutes at room temperature with antibodies to human IFNAR-1 (rabbit polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA), IFNAR-2b (rabbit polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and IFNAR-2c (monoclonal antibody, Dr E. Croze, Berlex Biosciences, Richmond, CA) subunits. Finally, a peroxidase complex (IL Immunologic, Duiven, The Netherlands) for IFNAR-1 and IFNAR-2b, or standard streptavidin-biotinylated alkaline phosphatase (IL Immunologic, Duiven, The Netherlands) for IFNAR-2c, were used according to the manufacturer's recommendations to visualize the bound antibodies. Negative controls for the immunohistochemistry included: 1) omission of the primary antibody; and 2) preabsorption of the antibody for IFNAR-2b with the respective immunizing receptor peptide.

Cell proliferation assay

After trypsinization, the cells were plated in 1 ml of medium in 24-well plates at a density of 5×10^3 cells/well. The plates were then placed in a 37°C, 5% CO₂ incubator. Three days later

the cell culture medium was replaced with 1ml/well medium containing various concentrations (0, 1, 5, 10, 50, 100, 1000 IU/ml) of IFN- α or IFN- β (Serono Inc.). Quadruplicates of each treatment were performed. Plates were further incubated at 37°C and 5% CO₂. After 3 and 6 days of treatment cells were harvested for DNA measurement. Plates for 6 days were refreshed after 3 days and compounds were added again. Measurement of total DNA-contents, representative for the number of cells, was performed using the bisbenzimidazole fluorescent dye (HoechstTM 33258) (Boehringer Diagnostics, La Jolla, CA), as previously described (22).

To evaluate the specificity of the effects of IFN- β on cell growth, we exposed type I IFNs with anti-human IFN- β neutralizing antibody. Cells (5×10^3 cells/well) were plated in 24-well plates and after 3 days of incubation at 37°C and 5% CO₂, the medium containing IFN- β (0, 5, 50 IU/ml) or IFN- α (100 IU/ml) in the absence or in presence of anti-human IFN- β neutralizing antibody (4 μ g/ml), was changed. Plates were refreshed after 3 days and compounds were added again. After 6 days of treatment plates were collected for DNA measurement. Both IFNs, alone or in combination with anti-human IFN- β neutralizing antibody, have been incubated for 1 day at 4°C prior to be added to BON cells, in order to antagonize the IFN- β activity.

Apoptosis assays

After trypsinization BON cells were plated in 6-well plates at a density of 0.5×10^5 cells/well. These plates were placed in a 37°C, 5% CO₂ incubator. Three days later the medium was refreshed in the presence and absence (control group) of IFN- α or IFN- β (Serono Inc.) at the concentration of 100 IU/ml. After 1, 2 and 3 days of incubation cells were gently trypsinized and washed with ice-cold PBS. Cells were resuspended in 100 μ l of 1 x binding buffer (Nexins Research B.V., Hoeven, The Netherlands), and stained with 5 μ l of FITC-annexin V (25 μ g/ml) and 10 μ l of 7-amino-actinomycin D (7-AAD) (1 mg/ml). Cells were incubated for 15 min on ice in the dark. Every sample was diluted with 385 μ l of 1 x binding buffer and immediately analyzed by FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium). At least 20000 cells were detected for each sample. With the use of CellQuest Pro Software, three subsets of cells, based on intensity of staining with annexin and 7-AAD, were identified: annexin⁻/7-AAD⁻ (live cells), annexin⁺/7-AAD⁻ (early apoptotic cells) and annexin⁺/7-AAD⁺ (late apoptotic and necrotic cells) (23). Subsequently, the percentage of each population was calculated. Annexin and binding buffer were obtained from Nexins Research (Kattendijke, The Netherlands), while 7-AAD from Becton and Dickinson.

Apoptosis was further confirmed by the analysis of the DNA fragmentation. After plating 5×10^4 cells/well on 24 well plates, cells were incubated at 37°C. Three days later the cell culture medium was replaced with 1ml/well medium containing various concentrations (0, 1, 5, 10, 50, 100, 1000 IU/ml) of IFN- α or IFN- β (Serono Inc.). Quadruplicates of each treatment were performed. After an additional incubation of 1 day, apoptosis was assessed using a commercially available ELISA kit (Cell Death Detection ELISA^{Plus}, Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used, as previously described (24).

Cell cycle analysis

Cells (0.8×10^6) were plated in 75cm² flasks. After 3 days medium was changed with fresh medium (control group) and with fresh medium plus IFN- α or IFN- β (Serono Inc.) at the concentration of 100 IU/ml. After 1, 2 and 3 days of incubation (with a confluence of about 60-70%), cells were harvested by gentle trypsinization, washed with cold PBS (calcium and magnesium free), and collected by centrifugation. For cell cycle analysis, approximately 10^6 cells were re-suspended in 200 μ l of PBS and fixed in 70% ice-cold ethanol with an overnight incubation at -20°C. After brief centrifugation, cells were washed once with PBS and incubated for 30 minutes at 37°C in PBS containing 40 μ g/ml of propidium iodide (Sigma Aldrich, The Netherlands) and 10 μ g/ml of DNase-free RNase (Sigma Aldrich, The Netherlands). For each tube 20000 cells were immediately measured on a FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) using CellQuest Pro Software.

Chromogranin A determinations

BON cells (5×10^3 cells/well) were cultured in 24-well plates and placed in a 37°C, 5% CO₂ incubator. Three days later the culture medium was replaced with 1ml/well medium containing various concentrations of IFN- α or IFN- β (Serono Inc.) (0, 1, 5, 10, 50, 100 IU/ml). Plates were further incubated at 37°C and 5% CO₂, refreshed after 3 days and compounds were added again at escalating concentrations. After 6 days of treatment the supernatant and the plates were collected for chromogranin A and DNA measurements, respectively. Human chromogranin A was measured in the conditioned media by a solid-phase two-site immunoradiometric assay based on monoclonal antibodies that bind to two distinct contiguous epitopes within the 145-245 region of chromogranin A (Chromogranin A-RIA CT, CIS bio international, Gif-sur-Yvette, France), according to the manufacturer procedures (25).

Effects of two different preparations of IFN- β -1a on cell growth and chromogranin A production.

After trypsinization, the cells were cultured in 24-well plates at a density of 5×10^3 cells/well. The plates were then placed in a 37°C, 5% CO₂ incubator. Three days later the cell culture medium was replaced with 1ml/well medium containing various concentrations (0, 1, 5, 10, 50, 100 IU/ml) of IFN- β -1a from 2 different preparations: Serono Inc. and GIBCO Brl. Plates were refreshed after 3 days and compounds were added again. After 6 days of treatment cells and conditioned medium were harvested, as indicated above, for DNA and chromogranin A measurement.

Statistical analyses

All experiments were carried out at least three times and gave comparable results. For statistical analysis GraphPad Prism™ 3.0 (GraphPad Software, San Diego, USA) was used. Relative 50% growth-inhibition concentrations (IC₅₀) and maximal inhibitory effect were calculated using nonlinear regression curve-fitting program. The comparative statistical evaluation among groups was firstly performed by ANOVA test. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. The unpaired Student t-test was chosen to analyze the differences in concentration-effect curves (IC₅₀ and maximal inhibitory effect) and effects in cell cycle modulation between different types or preparations of IFNs, and the differences of the growth inhibitory effects of IFNs after 3 and 6 days of treatment. Correlation analyses were performed using Pearson's coefficients. In DNA fragmentation analyses, the means of quadruplicates for each treatment with the same dose of IFN- β have been correlated to the corresponding means of quadruplicates in the corresponding plates for the cell proliferation assay after 6 days of treatment with IFN- β . In addition, in one experiment DNA measurements after 6 days of treatment with IFN- β have been correlated to the corresponding chromogranin A concentrations, evaluated in the corresponding conditioned medium. In all analyses, values of $p < 0.05$ were considered statistically significant. Data are reported as mean \pm SEM.

Results

Expression of type I IFN receptors mRNA and proteins in BON cells

Since the activity of type I IFNs is modulated by a common receptor, we analyzed the expression of IFNAR-1 and IFNAR-2 (total, short and long form) mRNA in BON cells by quantitative RT-PCR (fig.1A).

Using sequence specific primers against the type I IFN receptor subunits, we detected the presence of IFNAR-1, IFNAR-2 total, IFNAR-2b and IFNAR-2c mRNA. IFNAR-1 mRNA expression, normalized for the amount of the housekeeping gene HPRT, was higher than IFNAR-2 total. Among IFNAR-2 subunits, IFNAR-2c was the form expressed at a relatively low level.

Specific immunoreactivity for IFN receptor subunits (IFNAR-1, IFNAR-2b and IFNAR-2c) was found in BON cells at immunocytochemistry (fig.1B-D). It was strongly positive for IFNAR-1 on the membrane and cytoplasm (fig.1B), moderately positive for IFNAR-2b on the membrane and cytoplasm (fig.1C) and weakly positive for IFNAR-2c on the membrane (fig.1D). Therefore, quantitative RT-PCR study demonstrated the presence of type I IFN receptor subunit transcripts and immunocytochemistry confirmed the presence of activable receptor proteins on BON cell membrane.

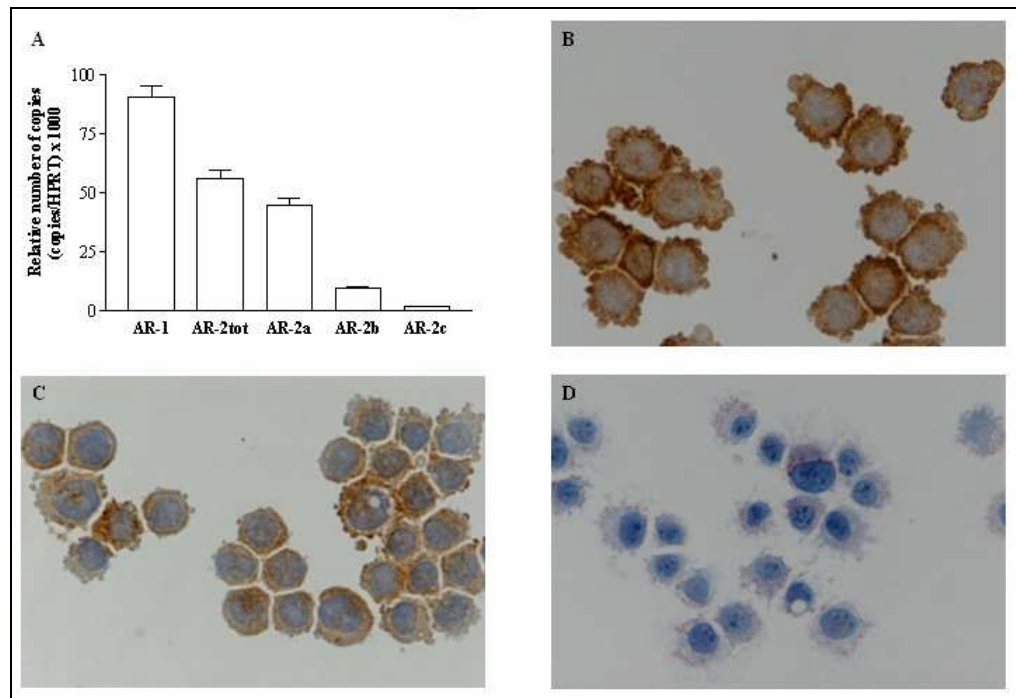


Figure 1. A: Relative expression of type I IFN receptor (AR-1, AR-2 total, AR-2b, AR-2c) mRNA in the human carcinoid BON cell line, evaluated by quantitative RT-PCR. The amount of IFN receptor mRNA was calculated relative to the amount of HPRT mRNA and is given in arbitrary units. The soluble form of IFNAR-2a subunit was determined indirectly by subtracting IFNAR-2b and IFNAR-2c from IFNAR-2 total. Values represent the mean \pm SEM. B-D: Immunocytochemical detection of IFNAR-1 (B), IFNAR-2b (C) and IFNAR-2c (D) receptors in human BON carcinoid cells. A strong IFNAR-1 immunostaining is evident in the membrane and cytoplasm. A moderate positivity for IFNAR-2b was detected in the membrane and cytoplasm, whereas a weak immunostaining is evident for IFNAR-2c along the cell membrane. Magnification, x 400.

Effects of type I IFNs on cell growth

After 6 days of incubation, IFN- α and IFN- β significantly suppressed the growth of BON cells in a dose-dependent manner, with an IC₅₀ of 44 IU/ml and 8 IU/ml, respectively (fig.2A-B). The growth-inhibitory effect of IFN- β was significantly more potent than that of IFN- α , as shown by the higher maximal inhibition of proliferation induced by IFN- β , compared with IFN- α

after 3 ($74.8\% \pm 2$ and $16.3\% \pm 1.3$, respectively, $p < 0.0001$; fig.2A) and 6 days ($97.6\% \pm 4.3$ and $25.8\% \pm 2.6$, respectively, $p < 0.0001$; fig.2B) of treatment, as well as by the lower $\log IC_{50}$ of IFN- β compared with IFN- α after 3 (1.1 ± 0.1 and 1.6 ± 0.1 , respectively, $p < 0.05$) and 6 days (0.9 ± 0.1 and 1.6 ± 0.2 , respectively, $p < 0.0001$). Note that after 6 days of incubation IFN- β induced a statistically significant cell growth inhibition already at very low concentrations (1 IU/ml). The antiproliferative effects of IFN- α and IFN- β were time-dependent. In fact, the maximal inhibition of cell proliferation, induced by both cytokines, resulted to be higher after 6 days compared with 3 days of incubation (IFN- α : $p < 0.05$, IFN- β : $p < 0.0001$). No statistically significant difference was observed between the values of IC_{50} of inhibition after 3 and 6 days for either IFN- α or IFN- β .

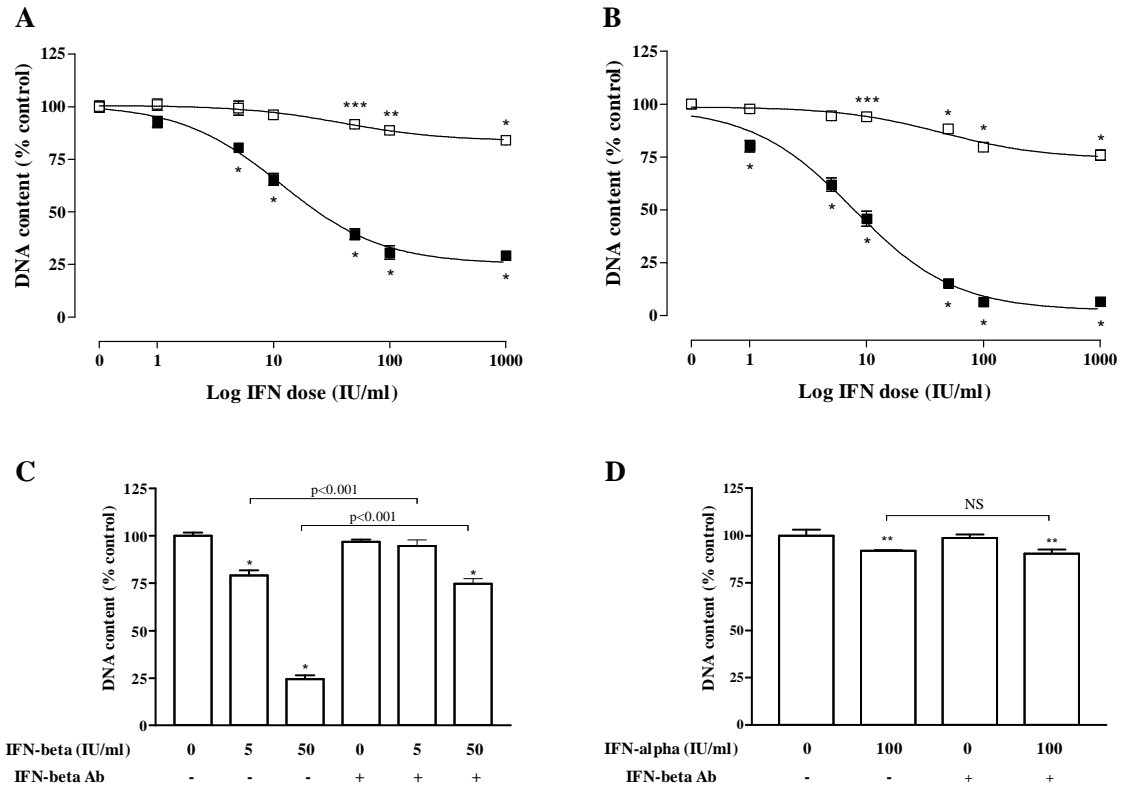


Figure 2. A-B: Dose-dependent inhibition of cell proliferation after 3 (A) and 6 (B) days of treatment with IFN- α and IFN- β in BON cell line. Measurement of total DNA-contents was performed using HoechstTM 33258. The mean DNA contents in controls were: 3278 ng/well (IFN- α , 3 days), 3592 ng/well (IFN- β , 3 days), 8941 ng/well (IFN- α , 6 days), 8497 ng/well (IFN- β , 6 days). : IFN- α ■: IFN- β . *: $p < 0.001$, **: $p < 0.01$, ***: $p < 0.05$ vs control. **C-D:** Effects of IFN- β (C) and IFN- α (D) on cell growth, alone and following pre-absorption with neutralizing antibodies against IFN- β (IFN-beta Ab). *: $p < 0.001$, **: $p < 0.05$ vs relative controls. Measurement of total DNA-contents was performed using HoechstTM 33258. Values are expressed as the percentage of control (untreated cells) and represent the mean \pm SEM of at least 3 independent experiments in quadruplicate.

To clarify the specificity of action of IFN- β we evaluated the effects on cell growth after a pre-absorption of IFN- α and IFN- β with neutralizing antibodies against IFN- β . As shown in fig.2C, the anti-tumor activity of 50 IU/ml IFN- β was significantly weakened by immuno-neutralization, whereas the effect of 5 IU/ml IFN- β was completely abolished. Moreover, these antibodies were unable to counteract the inhibitory effects of IFN- α on cell growth (fig.2D).

Effects of type I IFNs on apoptosis

The effects of IFN- α (100 IU/ml) and IFN- β (100 IU/ml) on the induction of apoptosis in BON cells were examined by flow cytometry. Using annexin V and 7-AAD, 2-color flow cytometric analysis detected 3 populations: viable, early apoptotic, and both late apoptotic and necrotic cells. Percentages of these populations, compared to the untreated control after 1, 2 and 3 days of treatment with IFN- α or IFN- β are shown in fig.3A, B, C. We observed (fig.3A) a significant decrease in the fraction of viable cells during the incubation with IFN- β (1, 2 and 3 days: $p < 0.01$). The percentage of early apoptotic cells significantly increased after 1, 2 and 3 days (all $p < 0.01$) of treatment with IFN- β (fig.3B). In the same way, IFN- β induced a significant increase ($p < 0.01$) in the fraction of late apoptotic/necrotic cells (fig.3C). It is noteworthy that, after 2 days of treatment with IFN- β (100 IU/ml) about 20% of cells showed signs of apoptosis or necrosis, which is 2.3 times higher than the control. In contrast, IFN- α did not significantly modify the fractions of life cells, early apoptotic and late apoptotic/necrotic cells in comparison to the untreated group.

During the treatment with IFN- α and IFN- β the measurement of the DNA fragmentation was further used to investigate the effect on the apoptosis (fig.4A-B). After 1 day of incubation, IFN- α had no remarkable stimulatory effects on DNA fragmentation at any concentration up to 1000 IU/ml in BON cell line. On the other hand, a dose-dependent induction of apoptosis was observed after IFN- β treatment, with a maximal increase of DNA fragmentation of about 3 times compared to the untreated control in BON cells. This cytokine stimulated apoptosis already at very low concentrations (1 IU/ml). These data were also confirmed by morphological observations. Only the treatment with IFN- β induced morphological alterations consistent with apoptosis, such as cell shrinkage, picnotic nucleus and detachment from the plate after 1-3 days (not shown). The inhibitory effects of IFN- β on the cell growth of BON cells appeared to be mainly due to an early pro-apoptotic activity, as shown by the highly significant positive correlation between the cell proliferation inhibition after 6 days of treatment and DNA fragmentation variation after 1 day of incubation ($r^2 = 0.82$, $p < 0.001$; fig.4C).

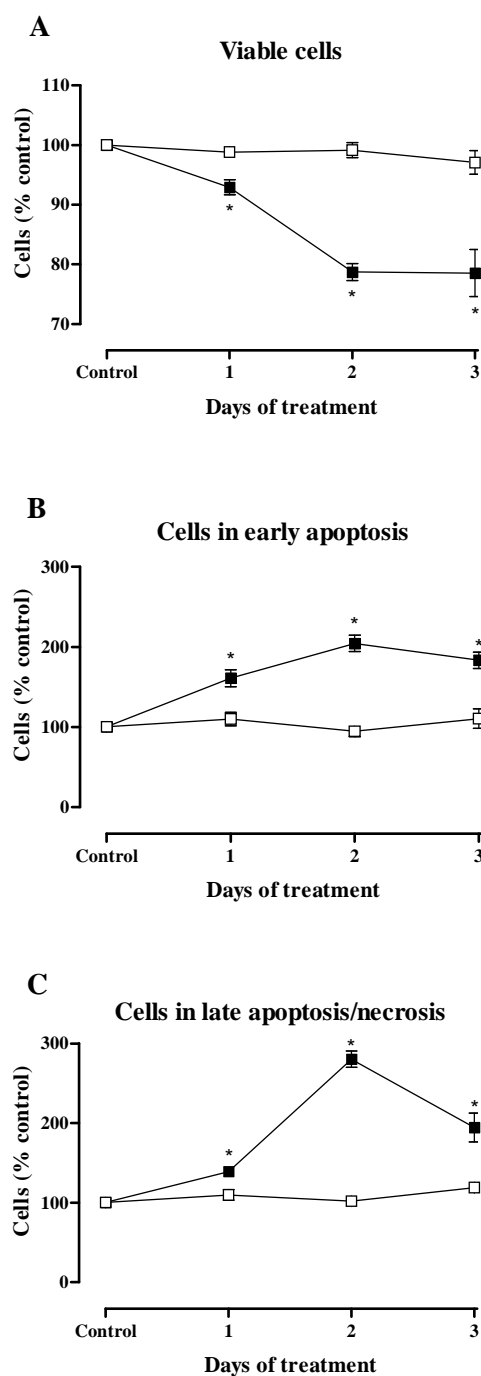


Figure 3A-C: Effects of IFN- α (100 IU/ml) and IFN- β (100 IU/ml) on the induction of apoptosis in BON cells, evaluated by flow cytometry for annexin V and 7-AAD.

Values of viable cells, early apoptotic cells and late apoptotic/necrotic cells are expressed as percentage compared to the untreated control. Data are reported as mean \pm SEM. Control values have been set to 100%. The mean percentages \pm SEM of viable cells in controls were: 89.2% \pm 1.3 (1 day), 88.1% \pm 0.9 (2 days), 85.9% \pm 1.3 (3 days). The mean percentages \pm SEM of early apoptotic cells in controls were: 4.5% \pm 0.2 (1 day), 4.7% \pm 0.9 (2 days), 4.2% \pm 0.4 (3 days). The mean percentages \pm SEM of late apoptotic/necrotic cells in controls were: 3.7% \pm 0.2 (1 day), 3.3% \pm 0.5 (2 days), 7.4% \pm 1.7 (3 days).

□: IFN- α

■: IFN- β (Serono).

*: $p < 0.01$ vs control.

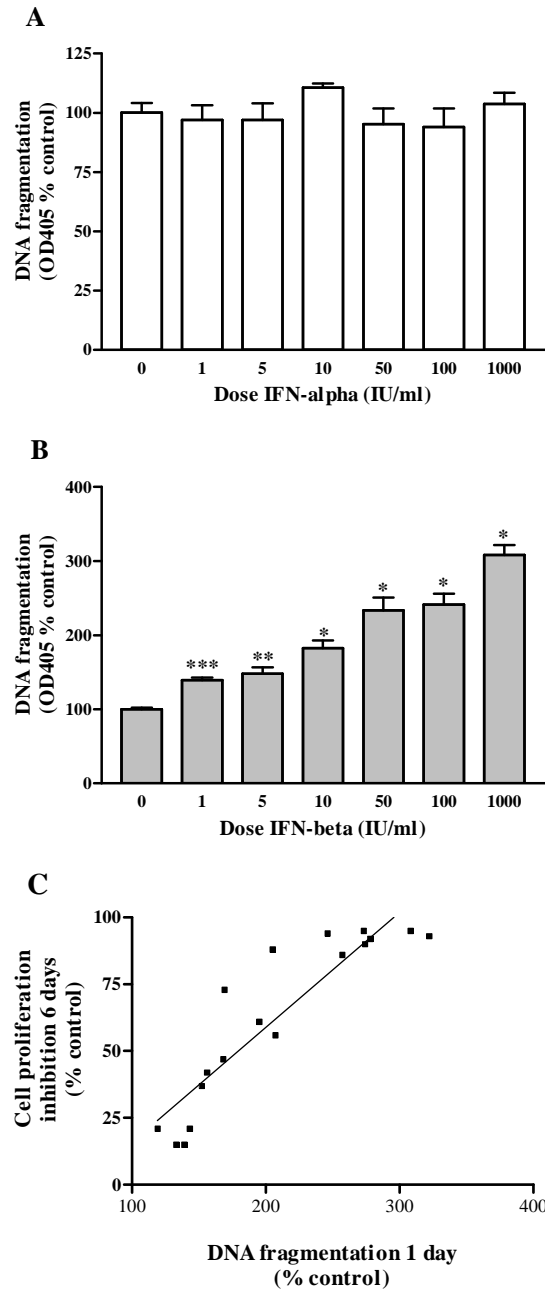


Figure 4A-B: DNA fragmentation after 1 day of treatment of BON cell line with IFN- α (A) and IFN- β (B). Values are absorbance units and are expressed as percent control. Data are the mean \pm SEM. *:p<0.001, **:p<0.01, ***:p<0.05 vs control.

C: Correlation between DNA fragmentation and cell proliferation inhibition after IFN- β treatment in BON cell line. Data correspond to the means of four wells for each treatment point with IFN- β in DNA fragmentation analysis after 1 day (on the abscissa) and to the means of quadruplicate for each treatment point in the respective plates for cell proliferation assay after 6 days of treatment with IFN- β (on the ordinate).

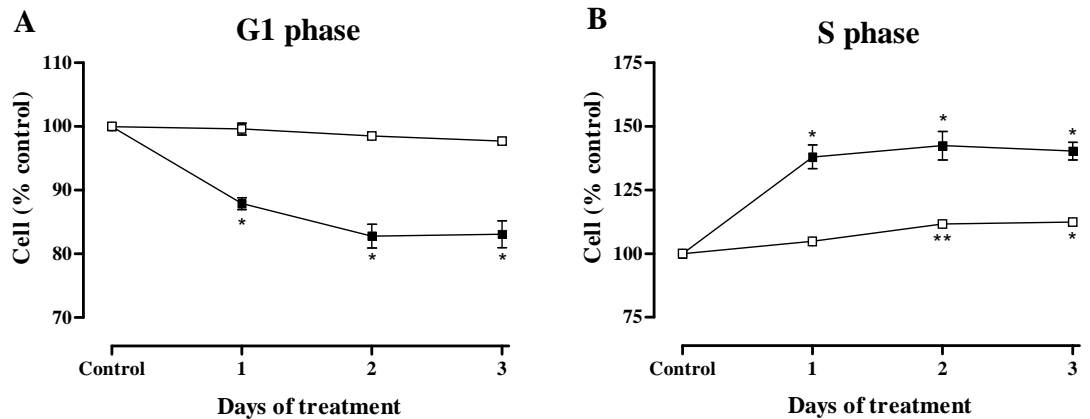
Effects of type I IFNs on the cell cycle

We also evaluated the effect of treatment with IFN- α (100 IU/ml) and IFN- β (100 IU/ml) on cell cycle phase distribution after 1, 2 and 3 days of incubation (fig. 5).

IFN- α treatment induced a slight, but significant accumulation of cells in S phase after 2 (p<0.05) and 3 days (p<0.001) of treatment compared to the control. In the same way, the incubation with IFN- β increased the fraction of BON cells in the S phase of the cell cycle already after 1 day (p<0.001 after 1,

2 and 3 days), whereas the proportion of cells in G₁ phase decreased in comparison with the control ($p < 0.001$ after 24h, 48h and 72h). These data suggested that BON cells in S phase failed to transit into G₂ and M phases efficiently and exhibited a prolonged stay in S phase after treatment with type I IFNs. Of note, the cell cycle arrest induced by IFN- β was more potent than that of IFN- α , as showed by the statistically significant difference in percentage of cells in S phase compared to the control after 1, 2 and 3 days of incubation with IFN- β and IFN- α (all $p < 0.001$).

Figure 5: Cell cycle distribution after 1, 2 and 3 days of incubation with 100 IU/ml IFN- α and 100 IU/ml IFN- β in BON cells. Data are expressed as mean \pm SEM of the percentage of cells in the different phases, compared with untreated control cells. Control values have been set to 100%. The mean percentages of control cells in G₁ phase were: 60% \pm 0.8 (1 day), 64.8% \pm 0.9 (2 days), 65.4% \pm 0.6 (3 days). The mean percentages of control cells in S phase were: 23.9% \pm 0.9 (1 day), 20.6% \pm 0.9 (2 days), 21% \pm 0.6 (3 days). \square : IFN- α \blacksquare : IFN- β . *: $p < 0.001$, **: $p < 0.05$ vs control.



Biochemical effects of type I IFNs.

The chromogranin A levels in the media, collected from BON cells during the interval 3-6 days of incubation with IFN- β , were significantly suppressed, also using low dose (fig. 6B). This effect was dose dependent, with IC₅₀ values of 6 IU/ml (comparable with the IC₅₀ of IFN- β on cell proliferation inhibition). On the other hand, IFN- α (fig. 6A) induced only a slight suppression of chromogranin A even at high dose (100 IU/ml). It is interesting to observe that, chromogranin A values, detected after 6 days of treatment with IFN- β , highly correlated with the relative values of DNA quantification ($r^2 = 0.98$, $p < 0.0001$).

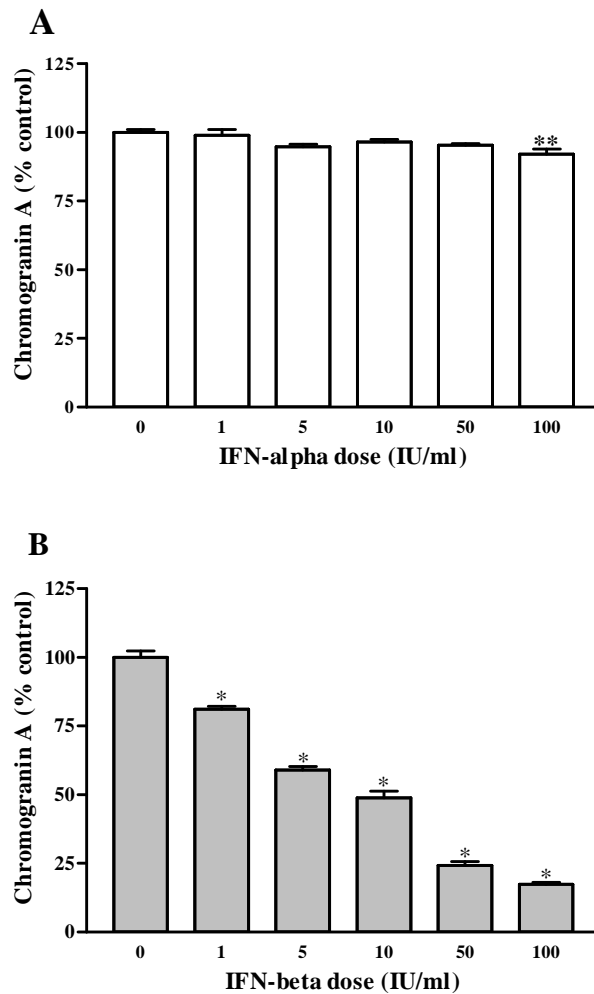


Figure 6: Effect of IFN- α (**A**) and IFN- β (**B**) on the production of chromogranin A in human BON cells after 6 days of incubation. Plates were refreshed after 3 days of treatment and compounds added again. Values are expressed as the percentage of control chromogranin A release and represent the mean \pm SEM. The mean chromogranin A levels in controls were: 3554 ng/well (panel A), 3759 ng/well (panel B). *: $p < 0.001$, **: $p < 0.01$ vs control.

Effects of two different preparations of IFN- β -1a on cell growth and chromogranin A production.

The potencies of IFN- β -1a preparations from two different sources (Serono Inc. and GIBCO Brl) on cell proliferation and chromogranin A production were superimposable (IC_{50} on cell growth: 6 IU/ml for both preparations; IC_{50} on chromogranin A: 4 IU/ml and 3 IU/ml, respectively). For IFN- β Serono and IFN- β GIBCO no statistically significant difference was observed between the values of $\log IC_{50}$ (on cell growth: 0.79 ± 0.04 and 0.75 ± 0.06 , respectively; on chromogranin A: 0.65 ± 0.08 and 0.44 ± 0.12 , respectively) and maximal inhibition of proliferation (on cell growth: $98\% \pm 1.6$ and $99\% \pm 2.7$, respectively; on chromogranin A: 86 ± 2.8 and 85 ± 3.4 , respectively).

Discussion

IFN- α is currently used as a therapeutic agent in several malignancies, including GEP-NETs (14-16,26-29). This cytokine belongs to the class of type I IFNs (e.g. IFN- α , IFN- β , IFN- ω , IFN- κ and IFN- τ), which modulate a variety of biological responses through the activation of a common receptor, composed by two subunits: IFNAR-1 and IFNAR-2 (30,31). IFNAR-1 is considered the signaling subunit and it exists as the full chain. The IFNAR-2 is the subunit responsible for the interaction with the ligand. There are three forms of IFNAR-2, which are differentially spliced products of the same gene, e.g. the soluble (IFNAR-2a), short (IFNAR-2b) and long (IFNAR-2c) form (26,31,32). The chain IFNAR-2c with its long cytoplasmic domain and the IFNAR-1 subunit constitute the predominantly active form of the type I IFN receptor complex. IFNAR-2c is capable of binding ligand, but with a lower affinity (20-fold less) than the dimeric IFN receptor complex itself (33). Therefore, both receptor chains are required to form a high affinity binding site and initiate signal transduction. The short form is able to bind type I IFNs, but does not couple to signal transduction because it lacks the signal transducing tail of IFNAR-2c (34). The soluble form may act as a regulator of free IFNs and, depending on concentration, lead to the neutralization or enhancement of IFN bioactivity (35,36).

Whereas the role of IFN- α has been extensively studied, the effect of other type I IFNs on neuroendocrine cell proliferation has not been evaluated. IFN- β is a multifunctional cytokine binding to the same receptor of IFN- α , but with higher affinity (37). It seems to be an essential mediator not only for the host defense in the innate immune responses against microbial infections, but also for a host defense system against oncogenesis (28). Few studies showed that IFN- β has greater antitumor effects than IFN- α , via the activation of apoptosis (17-21). Therefore, IFN- β represents a promising drug in the treatment of cancer.

In the present study, we compared the anti-tumor effects of IFN- β -1a and IFN- α -2b in human GEP-NETs, as well as the mechanisms that are involved in the growth inhibition. The expression of mRNA and protein for the active subunits of type I IFN receptor (IFNAR-1 and IFNAR-2c) was detected by quantitative RT-PCR and immunocytochemistry in the human BON cell line. Both IFNs showed an inhibitory effect on the cell proliferation of these cells, but the antitumor activity of IFN- β -1a was significantly higher than that of IFN- α -2b. This difference could be explained by the potent pro-apoptotic activity of IFN- β -1a, as shown by the increase in apoptotic cells, evaluated by flow cytometry and the increase in DNA fragmentation. On the other hand,

IFN- α -2b did not stimulate apoptosis in BON cells, but it was able to induce a cell cycle arrest with an accumulation of the cells in S phase. These results are consistent with previous studies, which demonstrated a delay in S-G₂/M phase transit of the cell cycle after treatment with IFN- α in the human GEP-NETs cells (38,39). The present study shows that apart from the potent induction of apoptosis, IFN- β -1a can induce a cell cycle arrest as well, resulting in increasing the fraction of cells in the S phase. Moreover, this latter effect was significantly more potent and earlier than that of IFN- α -2b. The specificity of the effects of IFN- β on BON cell growth is shown by the abolishment of the inhibitory effect of IFN- β after incubation with neutralizing antibodies against IFN- β in BON cells.

Although, IFN- α and IFN- β interact with the same receptor, the induction of differential pathways can be explained by the differences in the structure between both cytokines (40,41), generating differential interactions with the same receptor. In fact, although both IFNs induce tyrosine phosphorylation of the receptor subunits, IFN- β , but not IFN- α , induces the association of IFNAR-1 and IFNAR-2c chains, indicating that the specificity of signaling for distinct type I IFN subtypes could be established by differential conformation of the receptor complex (34,42). Moreover, it was demonstrated that IFN- α can trigger survival pathways in human tumor cells that could, at least in part, explain its poor effects on apoptosis onset if compared with that one caused by IFN- β (43). On the basis of these results, studies on the pathways activated by the two IFNs isoforms are warranted. However, we cannot exclude that apoptosis induced by IFN- β was likely due to the strong lack of transition of BON cells from S to G₂/M phase of cell cycle. In fact, apoptosis could arise as a consequence of inefficient cell cycle progression, culminating in apoptosis (44).

In addition, we evaluated the effects of IFN- α and IFN- β on chromogranin A production. Chromogranin A is the most sensitive and specific marker in GEP-NETs (45,46) and BON cells are able to release this substance. In patients, circulating chromogranin A levels are dependent by several factors: tumor mass, secretory activity and granule density of tumor cells (45,46). This may render the use of chromogranin A in monitoring the response of tumor mass during medical treatment difficult. In fact, several drugs (e.g. somatostatin analogs) induced a decrease in plasma chromogranin A levels, through an inhibitory effect on synthesis and secretion, without any effect on tumor growth (46). We detected a very potent dose dependent suppression of the chromogranin A levels in the conditioned media collected from BON cells in the interval 3-6 days of incubation with IFN- β , whereas the biochemical effect of IFN- α was minor and statistically present only at high dose. On the

basis of a strong positive correlation between chromogranin A concentrations and DNA quantification after 6 days of treatment with IFN- β , we hypothesize that the suppression of this tumor marker results from the decrease in the number of the cells, rather than from an inhibition in synthesis and secretion. This observation suggests that, in patients the determinations of plasma chromogranin A could give important information about the effect on tumor growth during the treatment of GEP-NETs with IFN- β , also considering that the estimation the total tumor mass in disseminated GEP-NETs is not easy (46).

Finally, our findings support the clinical attractiveness to use IFN- β in the treatment of GEP-NETs, considering that IFN- β inhibited cell proliferation and stimulated apoptosis already at very low concentrations (1-10 IU/ml). These concentrations can be achieved *in vivo*, since 12.3 IU/ml is the maximal IFN- β serum concentration reported in healthy subjects after subcutaneous administration of this cytokine (47,48). Translation of these findings into a clinical application is, however, not easy, considering the short half life of IFN- β . But new promising strategies (PEGylated form, carrier proteins like IFNAR-2a, or gene therapy) can improve pharmacokinetic and pharmacodynamic properties of IFN- β , bringing its real clinical application in cancer closer (36,49,50).

In conclusion, this is the first study showing that the antitumor activity of IFN- β in GEP-NETs is considerably more potent than IFN- α . This seems to be related to the fact that IFN- α induced cell cycle arrest only, whereas both apoptosis and cell cycle arrest were responsible for IFN- β -mediated growth suppression even at very low doses. These data provide rationales for future preclinical and clinical trials using IFN- β in GEP-NETs.

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CHAPTER III:

TYPE I INTERFERONS IN THE TREATMENT OF PANCREATIC CANCER: MECHANISMS OF ACTION AND ROLE OF RELATED RECEPTORS



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Abstract

Chemotherapy and radiotherapy have only a marginal role in the management of pancreatic adenocarcinoma.

We evaluated the role of type I interferons (IFNs) and their receptors in the regulation of cell growth in 3 human pancreatic adenocarcinoma cell lines (BxPC-3, MiaPaCa-2 and Panc-1).

The treatment with IFN- β showed a very potent inhibitory effect on the proliferation of BxPC-3 (IC₅₀: 14 IU/ml) and MiaPaCa-2 (IC₅₀: 64 IU/ml) after 6 days. The inhibitory effect of IFN- β was dose- and time dependent and significantly stronger than IFN- α . This antitumor effect is mainly modulated by the stimulation of apoptosis, which was earlier and more potent than IFN- α , although cell cycle arrest was induced as well. Panc-1 was the most resistant cell line to both IFNs, showing a stimulation of apoptosis only at very high doses, and cell cycle arrest at moderate dose. As determined by real time quantitative RT-PCR and immunocytochemistry, all 3 cell lines expressed type I IFN receptor (IFNAR-1 and IFNAR-2 subunits) mRNA and protein. The expression of active subunits (IFNAR-1 and IFNAR-2c) was significantly higher in BxPC-3 and mainly localized on the membrane, whereas in Panc-1 the staining for IFNAR-1 and IFNAR-2c was mainly cytoplasmatic and about 60-70% of cells were negative for IFNAR-2c

In conclusion, IFN- β is much more potent than IFN- α in its anti-proliferative effect on pancreatic cancer cell lines through the induction of an early apoptosis, particularly in BxPC-3. The relative high expression of IFNAR-1 and IFNAR-2c subunits in BxPC-3 and membranous distribution could explain the major sensitivity of this cell line to IFN treatment.

Introduction

Pancreatic adenocarcinoma is a highly aggressive malignancy (1). Surgery is the only curative therapy. Unfortunately, due to a lack of specific symptoms, limitations in diagnostic methods and the biologically aggressive nature of this tumor, only 5% to 15% of patients are surgical candidates at the time of the diagnosis (1). In this selected group of patients, adjuvant chemotherapy has a significant survival benefit but the 5-year survival of 21 % remains poor (2). The role of chemoradiotherapy in the management of pancreatic adenocarcinoma is unclear (3). However, it has been showed that interferon (IFN)- α in combination with adjuvant chemoradiotherapy improved overall 5-year survival rates of 55% despite advanced tumors resected (4).

In vitro and *in vivo* studies have showed the efficacy of type I IFNs (e.g. IFN- α , IFN- β , IFN- ω , IFN- κ and IFN- τ), in the treatment of several tumors (5-9). Although the antitumor effects of IFN- α have been studied in detail, those of IFN- β are not well clarified. IFN- β is a multifunctional cytokine binding the same receptor of IFN- α , but with higher affinity (10). It seems to be an essential mediator not only for the innate immune responses against microbial infections, but also for a host defense system against oncogenesis (6,11). Besides, few studies showed that IFN- β has greater antitumor effects than IFN- α (10, 12-16). On the basis of these observations, IFN- β represents a promising drug in the treatment of cancer.

Importantly, several chromosomal aberrations have been detected in pancreatic adenocarcinoma, including a frequent loss of chromosome arm 9p, observed in more than 80% of human pancreatic cancer (17). Together to the tumor-suppressor genes p16INK4a, p15INK4b and p14ARF also the IFN- α and IFN- β genes are located on chromosome 9p (18). Therefore, in relation to the defensive role of IFNs against tumors (11), the absence of IFNs expression may have an important role in the pathogenesis of pancreatic adenocarcinoma.

To further explore the possibilities of new medical treatments in pancreatic cancer, we evaluated in the present study the antitumor activity of IFN- α and IFN- β in 3 human pancreatic adenocarcinoma cell lines (BxPC-3, MiaPaCa-2 and Panc-1) and the role of their receptors in the responsiveness to type I IFNs.

Methods

Cell lines and culture conditions

The human pancreatic cell lines, BxPC-3, MiaPaCa-2 and Panc-1 were purchased from American Type Culture Collection. The cells were cultured in a humidified incubator

containing 5% CO₂ at 37°C. The culture medium consisted of RPMI 1640 supplemented with 10% FCS, penicillin (1x10⁵ U/l) and L-glutamine (2 mmol/l). Periodically, cells were confirmed as *Mycoplasma*-free. Cells were harvested with trypsin (0.05%), EDTA (0.02%) and resuspended in medium. Before plating, cells were counted microscopically using a standard hemocytometer. Trypan Blue staining was used to assess cell viability, and always exceeded 95%. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

Drugs and Reagents

Human recombinant IFN- α -2b (Roferon-A) was obtained from Roche (Mijdrecht, The Netherlands), while human recombinant IFN- β -1a was acquired from Serono Inc. (Rebif, Rockland, MA). Polyinosic:polycytidylic acid (poly I:C) was obtained from Sigma-Aldrich (Leiden, The Netherlands). All compounds were stored at -20°C, and the stock solution was constituted in distilled water according to the manufacturer instructions.

Cell proliferation assay

After trypsinization the cells were plated in 1 ml of medium in 48-well plates at a density of 5x10³ – 4x10⁴ cells/well, depending on the length of the incubation period. The plates were then placed in a 37°C, 5% CO₂ incubator overnight. The next day the cell culture medium was replaced with 1ml/well medium containing increasing concentrations (0-10000 IU/ml) of IFN α or IFN β . Quadruplicates of each treatment were performed. Plates were further incubated at 37°C and 5% CO₂. After 1, 3 and 6 days of treatment cells were harvested for DNA measurement, at approximately 70-80% confluence. Plates for 6 days were refreshed after 3 days and compounds were added again. Measurement of total DNA-contents, representative for the number of cells, was performed using the bisbenzimidazole fluorescent dye (HoechstTM 33258) (Boehringer Diagnostics, La Jolla, CA), as previously described (19).

Cell Death Detection (DNA fragmentation)

After plating 10⁴ – 4x10⁴ cells/well, depending on the length of the incubation period, on a 48 well plate cells were left overnight to adhere. The next day the cell culture medium was replaced with 1ml/well medium containing increasing concentrations (0-10000 IU/ml) of IFN α or IFN β . Quadruplicates of each treatment were performed. After an additional incubation of 1 and 3 days, apoptosis was assessed using a commercially available ELISA kit (Cell Death Detection ELISA^{Plus}, Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used, as previously described (20). Relative apoptosis was determined by a ratio of the average absorbance of the treatment wells to the average absorbance of the control wells. The data were corrected for the effect on cell number after 1 and 3 days of treatment.

Cell cycle analysis

Cells (1-4 x 10⁶), depending on the length of the incubation period, were plated in 75cm² culture flasks (Corning Costar, Amsterdam). After 1 day medium was changed with fresh medium (control group) and with fresh medium plus IFN- α or IFN- β at the concentration of 1000 IU/ml. Duplicates of each treatment were performed. After 1, 2 and 3 days of incubation, cells were harvested by gentle trypsinization and prepared for cell cycle determination using propidium iodide for DNA staining, as previously described (17). The stained cells were analyzed by FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) and CellQuest Pro Software. Cell cycle progression was measured with corresponding absorbances for G₀/G₁, S and G₂-M phases, whereas apoptosis was measured by quantifying the sub-G₀ peak.

Quantitative RT-PCR

The expression of type I IFN receptors (IFNAR-1, IFNAR-2 total, the short form IFNAR-2b, and the long form IFNAR-2c), IFN- β and housekeeping gene hypoxanthine-phosphoribosyl-transferase (HPRT) mRNA was evaluated by quantitative RT-PCR in all 3 pancreatic cancer cell lines, as previously described (17). Briefly, poly A⁺ mRNA was isolated using Dynabeads Oligo (dT)₂₅ (Dynal AS, Oslo, Norway) from cell pellets containing approximately 5x10⁵ cells or from cells previously plated on 48 well plates, as reported in poly I:C section. Complementary DNA (cDNA) was synthesized using the poly A⁺ mRNA in

a Tris-buffer together with 1mM of each deoxynucleotide triphosphate, 10 U RNase inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 40 μ l. This mixture was incubated for 1 h at 42°C. One fifth of the cDNA library was used for quantification of IFN receptors, IFN- β and HPRT mRNA levels. A quantitative PCR was performed by AmpliTaq Gold® DNA Polymerase and the ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Groningen, The Netherlands) for real-time amplifications, according to the manufacturer's protocol. Each sample was assayed in duplicate. The assay was performed using 15 μ l TaqMan® Universal PCR Master Mix (Applied Biosystems, Capelle aan de IJssel, The Netherlands), forward primer, reverse primer, probe and 10 μ l cDNA template, in a total reaction volume of 25 μ l. PCR amplification started with a first step for 2 min at 50°, followed by an initial heating at 95°C for 10 min, samples were subjected to 40 cycles of denaturation at 95°C for 15 sec and annealing for 1 min at 60°C.

The primer and probe sequences that were used for the detection of IFNAR-1, IFNAR-2 total, IFNAR-2b, IFNAR-2c and HPRT have been previously described (19). In addition, we also evaluated IFN- β mRNA expression in the present study using the following primers and probe:

IFN- β forward, 5'-CAGCAATTTTCAGTGTCTCAGAAGCT-3';

IFN- β reverse, 5'-TTCATCCTGTCCTTGAGGCAG-3';

IFN- β probe, 5'-FAM-TGTGGCAATTGAATGGGAGGCTTGAAT-TAMRA-3'.

All the primer and probe sequences were purchased from Biosource (Nivelles, Belgium).

The detection of HPRT mRNA was used for normalization of IFN receptor mRNA levels. Expression of IFNAR-2a mRNA, the soluble form of IFNAR-2 subunit, was determined indirectly by subtracting IFNAR-2b and IFNAR-2c from IFNAR-2 total. Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A⁺ mRNA preparation (because the IFN- β gene is intron-less), the cDNA reactions were also performed without reverse transcriptase and amplified with each primer-pair. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of HPRT and type I IFN receptors human cDNA was amplified in parallel with the cDNA samples.

Polyinosinic-polycytidylic acid (poly I:C) stimulation

Cells were plated at 6×10^4 - 8×10^4 cells/well, depending on the length of the incubation period, on 24 well plates and left to adhere overnight. After this period the medium was replaced. Poly I:C, double stranded RNA, a potent activator of IFN- β expression, was added at a concentration of 100 μ g/ml in quadruplicates and samples were collected after 2, 4, 6 and 24 hours of incubation, as described in the quantitative RT-PCR section. Samples were kept on ice and directly stored at -80°C.

Immunocytochemistry

Cytospin preparations of BxPC3, MiaPaCa-2 and Panc-1 cells were fixed with acetone for 10 minutes. After washing two times with PBS, the cells were incubated for 30 minutes at room temperature with antibodies to human IFNAR-1 (rabbit polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) and IFNAR-2c (monoclonal antibody, Dr E. Croze, Berlex Biosciences, Richmond, CA) subunits, and for overnight with antibodies to IFNAR-2b (rabbit polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Finally, a peroxidase complex for IFNAR-1 and IFNAR-2b, or standard streptavidin-biotinylated alkaline phosphatase (both from IL Immunologic, Duiven, The Netherlands) for IFNAR-2c, were used according to the manufacturer's recommendations to visualize the bound antibodies. Negative controls for the immunohistochemistry included: 1) omission of the primary antibody; and 2) preabsorption of the antibody for IFNAR-2b with the respective immunizing receptor peptide.

Statistical analyses

All experiments were carried out at least three times and gave comparable results. For statistical analysis GraphPad Prism™ 3.0 (GraphPad Software, San Diego, USA) was used. Fifty percent growth-inhibition concentrations (IC₅₀) and maximal inhibitory effect were

calculated using nonlinear regression curve-fitting program. The comparative statistical evaluation among groups was firstly performed by the ANOVA test. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. The unpaired Student t-test was chosen to analyze the differences in concentration-effect curves (IC_{50} and maximal inhibitory effect) and effects in cell cycle modulation between different types of IFNs, and the differences of the growth inhibitory effects of IFNs after 3 and 6 days of treatment. Correlation analyses were performed using Pearson's coefficients. In all analyses, values of $p < 0.05$ were considered statistically significant. Data are reported as mean \pm SEM. Statistical analysis was made after logarithmic transformation.

Results

Antiproliferative effects of type I IFNs

After 6 days of incubation, IFN- α and IFN- β significantly suppressed the growth of all three pancreatic cancer cell lines in a dose-dependent manner (Fig. 1), with a mean IC_{50} of 606 IU/ml and 14 IU/ml in BxPC-3, respectively; 1531 IU/ml and 64 IU/ml in MiaPaCa-2, respectively; 1250 IU/ml and 112 IU/ml in Panc-1, respectively.

The growth-inhibitory effect of IFN- β was significantly more potent than that of IFN- α , as shown by the higher maximal inhibition of proliferation induced by IFN- β compared with IFN- α ($96.7\% \pm 2$ and $72\% \pm 5.7$, respectively, $p < 0.0001$ in BxPC-3; $87.5\% \pm 3.2$ and $69.1\% \pm 6.1$, respectively, $p < 0.0001$ in MiaPaCa-2; $70.7\% \pm 1.4$ and $53\% \pm 5.7$, respectively, $p < 0.0001$ in Panc-1) after 6 days of treatment, as well as by the lower $\log IC_{50}$ of IFN- β compared with IFN- α (1.15 ± 0.06 and 2.78 ± 0.15 , respectively, $p < 0.00001$ in BxPC-3; 1.8 ± 0.07 and 3.18 ± 0.13 , respectively, $p < 0.00001$ in MiaPaCa-2; 2.05 ± 0.05 and 3.1 ± 0.17 , respectively, $p < 0.0001$ in Panc-1). It is interesting to note that in BxPC-3 and MiaPaCa-2 IFN- β induced a statistically significant cell growth inhibition already at very low concentrations (5-10 IU/ml).

In all 3 pancreatic cell lines the effects of IFN- α and IFN- β were time dependent. In fact, the maximal inhibition of cell proliferation, induced by both cytokines, resulted to be higher after 6 days compared with 3 days of incubation (both $p < 0.0001$ in BxPC3; $p < 0.005$ and $p < 0.0001$, respectively for IFN- α and IFN- β in MiaPaCa-2; both $p < 0.0001$ in Panc-1). In addition, in none of the cells a statistically significant difference was observed between the values of IC_{50} after 3 and 6 days of incubation with IFN- α or IFN- β .

The cell lines exhibited different sensitivities to the treatment, particularly with IFN- β . BxPC-3 resulted to be the most sensitive and Panc-1 the most resistant. The maximal inhibition of proliferation for IFN- β was higher in BxPC-3 than in MiaPaCa-2 ($p < 0.05$) and in Panc-1 ($p < 0.001$), while it was lower in Panc-1 compared with MiaPaCa-2 ($p < 0.01$). Similarly, the IC_{50} of IFN- β was significantly lower in BxPC-3 than in MiaPaCa-2 and Panc-1

(both $p < 0.001$), and higher in Panc-1 compared with MiaPaCa-2 ($p < 0.05$). The maximal inhibition of proliferation for IFN- α was higher in BxPC-3 compared with Panc-1 ($p < 0.05$), while no difference in IC_{50} values of IFN- α was observed between the 3 cell lines.

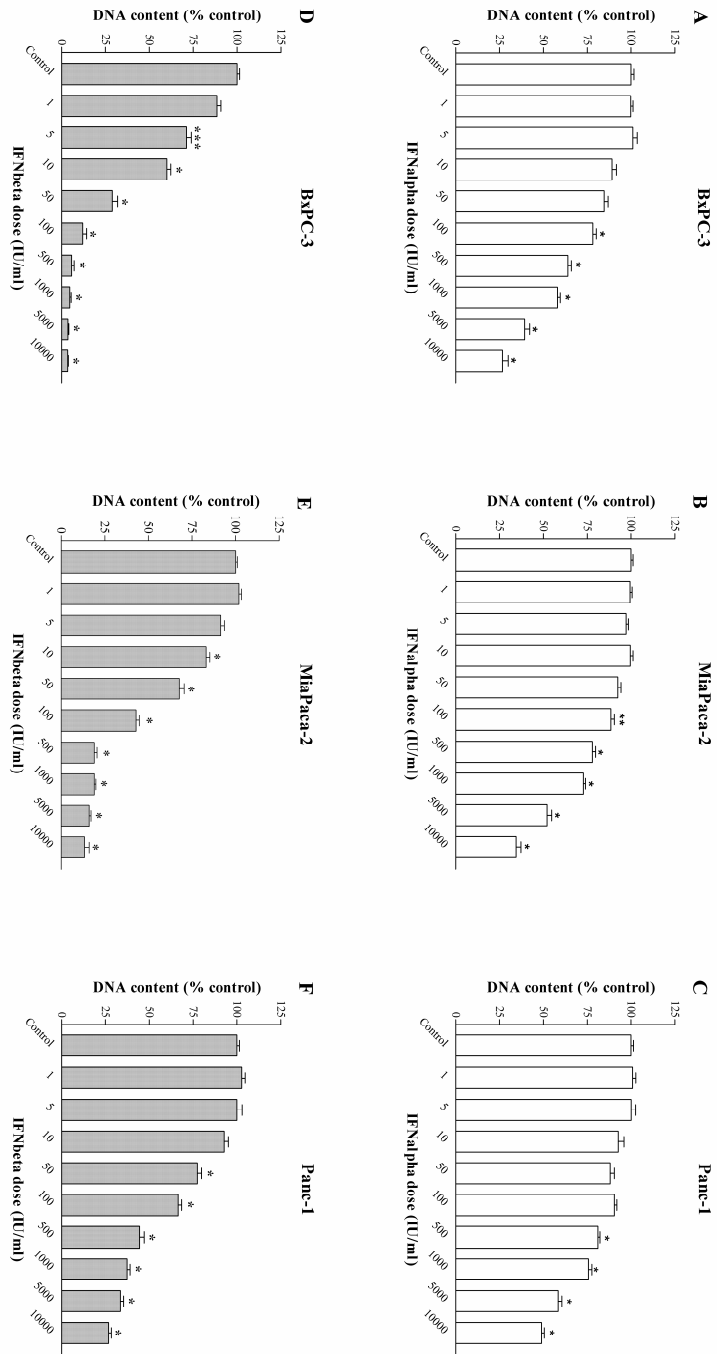


Figure 1: Effects of IFN- α (A, B, C) and IFN- β (D, E, F) on the cell proliferation, measuring total DNA-contents, representative for the number of cells, using HoechstTM 33258. Pancreatic cancer cell lines were incubated for 6 days without (control) or with the drugs indicated at different concentrations. Values are expressed as the percentage of control (untreated cells) and represent the mean \pm SEM of at least 3 independent experiments in quadruplicate. Natural logarithmic transformation was used to normalize the distributions for ANOVA. The mean DNA contents in controls were: 2260 ng/well (IFN- α , BxPC-3), 2430 ng/well (IFN- β , BxPC-3), 8562 ng/well (IFN- α , MiaPaCa-2), 8803 ng/well (IFN- β , MiaPaCa-2), 4224 ng/well (IFN- α , Panc-1), 4172 ng/well (IFN- β , Panc-1). *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$ vs. control.

Effects of type I IFNs on apoptosis

A crucial step in apoptosis is DNA fragmentation, a process that results from the activation of endonucleases, which degrade chromatin into smaller fragments. The measurement of DNA fragmentation was used to investigate the effect of treatment with IFN- α and IFN- β on apoptosis (Fig. 2 and 3).

After 1 day of incubation, IFN- α had no remarkable stimulatory effects on DNA fragmentation at any concentration up to 1000 IU/ml in all 3 cell lines, only at the dose of 10000 IU/ml IFN- α induced a significant increase in DNA fragmentation (Fig.2 A-C). On the other hand, a dose-dependent induction of apoptosis was observed after IFN- β treatment in BxPC-3 and MiaPaCa-2, already at a very low concentration (5-10 IU/ml), with a maximal increase of DNA fragmentation of about 3.5 times compared to the untreated control (Fig.2 D, E). In Panc-1 a stimulating effect on apoptosis has been observed only for high doses of IFN- β (≥ 500 IU/ml) (Fig. 2 F).

After 3 days of treatment with IFN- α an increase in DNA fragmentation has been detected in BxPC-3 and MiaPaCa-2 at a moderate to high dose (Fig 3 A,B). At this time the induction on apoptosis was still present after IFN- β with a maximal stimulation of about 4 and 10 times, compared to the control, respectively in MiaPaCa-2 and BxPC-3 (Fig 3 D, E). Also after 3 days of incubation the stimulating effects on apoptosis persisted in Panc-1 only at high doses of IFN- α (10.000 IU/ml, Fig. 3 C) and IFN- β (≥ 500 IU/ml, Fig. 3 F).

These data were also confirmed by morphological observations. In all three cell lines the treatment with IFN- β induced clear structural alterations consistent with apoptosis, such as cell shrinkage, picnotic nucleus and detachment from the plate after 1-3 days, also at very low doses in BxPC-3 cell line (not shown). These morphological changes are evident only at high doses of IFN- α treatment.

The inhibitory effects of IFN- β on the cell growth of BxPC-3 and MiaPaCa-2 cell lines appeared to be mainly due to an early pro-apoptotic activity, as shown by the highly significant positive correlation between the cell proliferation inhibition after 6 days of treatment and DNA fragmentation variation after 1 day ($r^2 = 0.95$, $p < 0.0001$, both for BxPC-3 and MiaPaCa-2) and 3 days of incubation ($r^2 = 0.95$, $p < 0.0001$, for BxPC3; $r^2 = 0.90$, $p < 0.0001$, for MiaPaCa-2). At early stage, apoptosis seems to be not involved in the antiproliferative effect of IFN- α on pancreatic cancer cells, in fact no significant correlation has been observed between cell proliferation inhibition after 6 days and DNA fragmentation variation after 1 day of treatment with IFN- α . Only after 3 days of treatment with IFN- α , we observed a positive correlation between DNA fragmentation variation and the 6 days cell

proliferation inhibition ($r^2 = 0.74$, $p < 0.0001$, for BxPC3; $r^2 = 0.71$, $p < 0.0001$, for MiaPaCa-2).

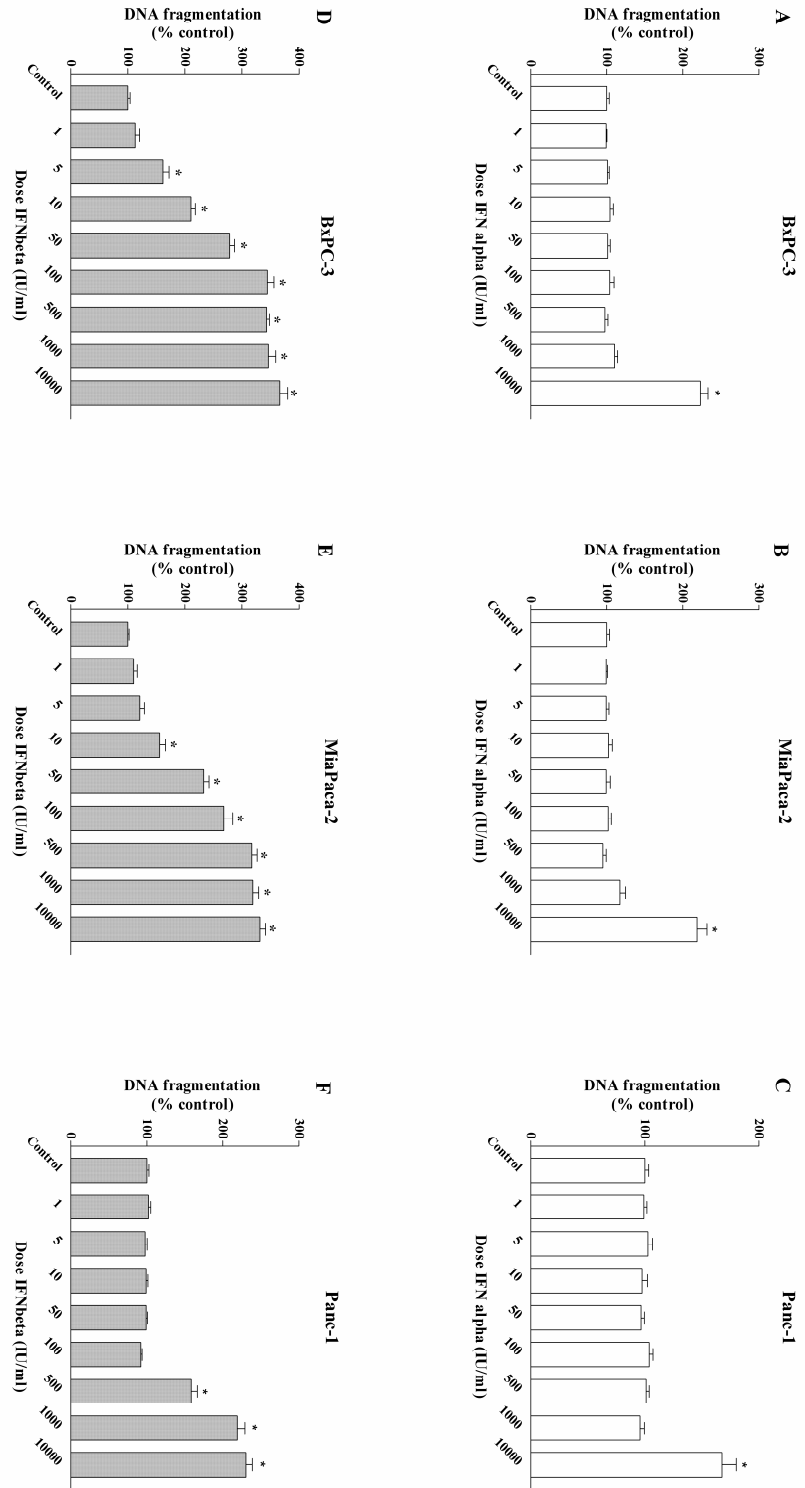


Figure 2: Effects of IFN-alpha (A, B, C) and IFN-beta (D, E, F) on the apoptosis (DNA fragmentation) in BxPC-3, Mia-PaCa-2 and Panc-1 cell lines. Pancreatic cancer cell lines were incubated for 1 day without (control) or with the drugs indicated at different concentrations. Values are absorbance units and are expressed as percent control. Data are the mean \pm SEM. * $P < 0.001$ vs. control.

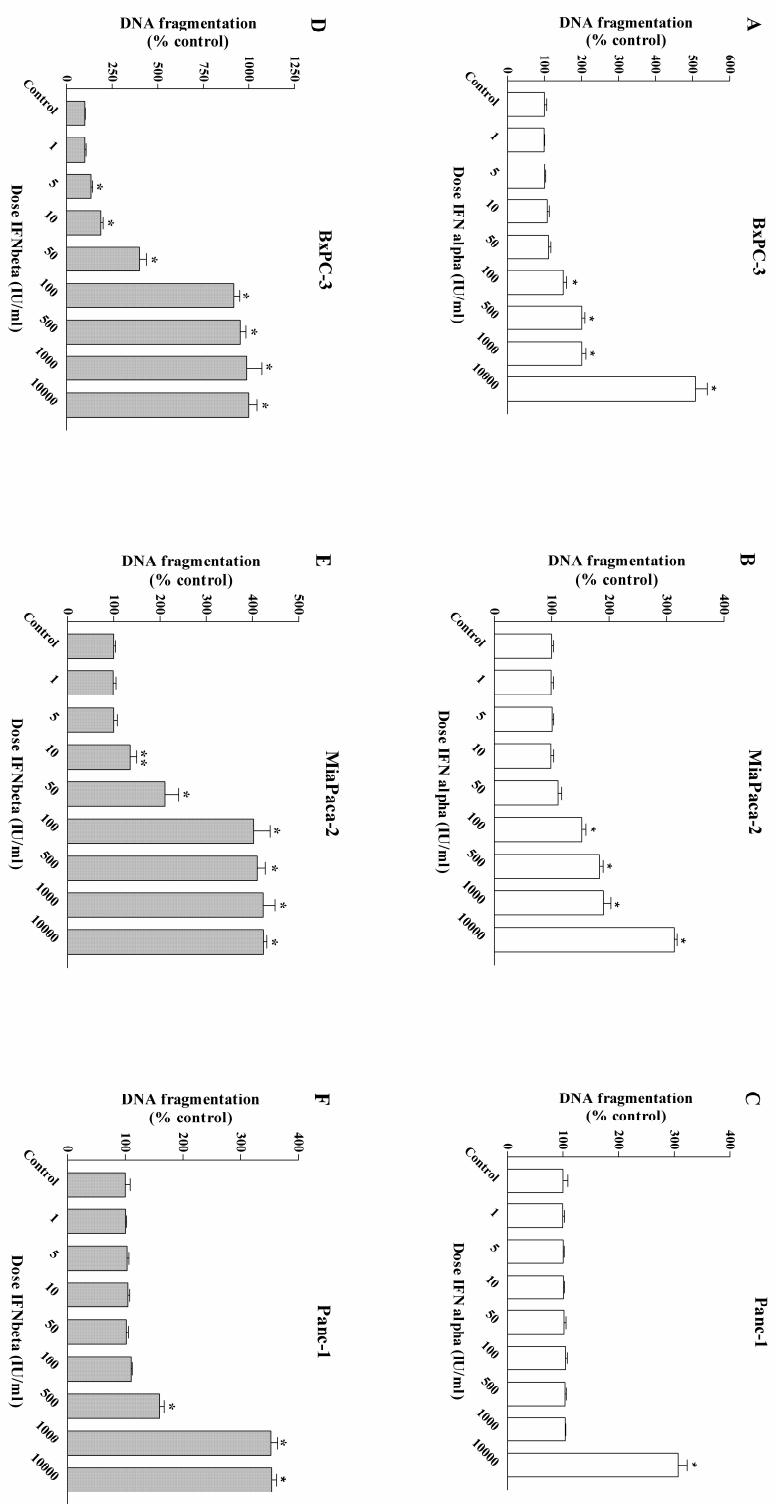


Figure 3: Effects of IFN-alpha (**A, B, C**) and IFN-beta (**D, E, F**) on the apoptosis (DNA fragmentation) in BxPC-3, MiaPaCa-2 and Panc-1 cell lines. Pancreatic cancer cell lines were incubated for 3 days without (control) or with the drugs indicated at different concentrations. Values are absorbance units and are expressed as percent control. Data are the mean \pm SEM. *, $P < 0.001$; **, $P < 0.01$

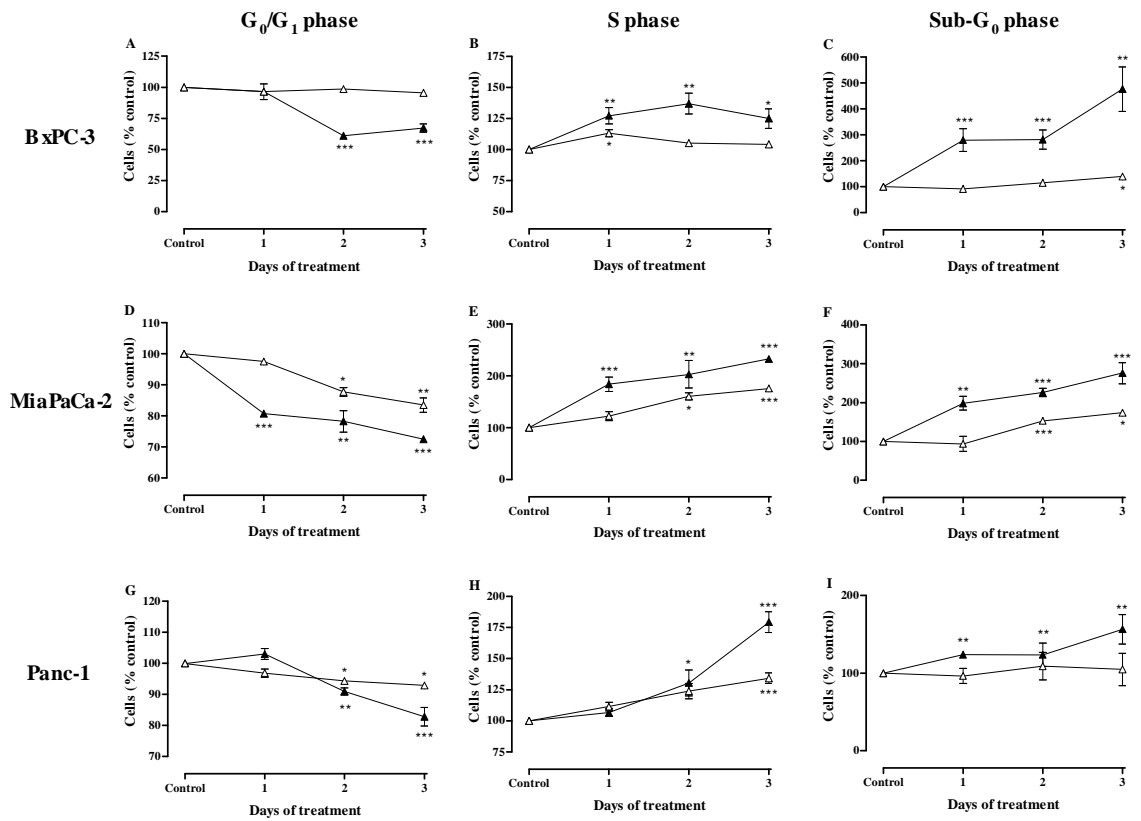


Figure 4: Cell cycle distribution after 1, 2 and 3 days of incubation with 1000 IU/ml IFN- α and 1000 IU/ml IFN- β in BxPC-3 (A-C), MiaPaCa-2 (D-F) and Panc-1 (G-I) cells. Data are expressed as mean \pm SEM of the percentage of cells in the different phases, compared with untreated control cells. Control values have been set to 100%.

△: IFN- α ▲: IFN- β . *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs control.

Effects of type I IFNs on the cell cycle

We also evaluated the effect of treatment with IFN- α (1000 IU/ml) and IFN- β (1000 IU/ml) on cell cycle phase distribution after 1, 2 and 3 days of incubation in BxPC-3, MiaPaCa-2 and Panc-1 (Fig. 4A-I).

IFN- α treatment induced a significant accumulation of all 3 cell lines in S phase compared to the control and a decrease in proportion of cells in G₀/G₁ phase in MiaPaCa-2 and Panc-1. In addition, the histograms of cell cycle revealed a late and slight increase in cells with subdiploid DNA content (sub-G₀ phase) only in BxPC-3 and MiaPaCa-2, confirming the induction of apoptosis after IFN- α treatment, as previously shown by the DNA fragmentation analyses. In the same way, the incubation with IFN- β increased the fraction of all 3 cell lines in the S phase of the cell cycle, whereas the proportion of cells in G₀/G₁ phase decreased in comparison with the control.

An accumulation of cells in sub-G₀ phase has been observed in BxPC-3, MiaPaCa-2 and Panc-1 after IFN- β treatment. These data suggested that pancreatic cancer cells in S phase failed to transit into G₂ and M phases and exhibited a prolonged stay in S phase after treatment with type I IFNs. The cell cycle arrest induced by IFN- β was more potent than that of IFN- α , considering that the percentage of cells in S phase compared to the control resulted to be higher after 3 days of incubation with IFN- β than after IFN- α (BxPC-3: $p < 0.05$, MiaPaCa-2: $p < 0.001$, Panc-1: $p < 0.001$).

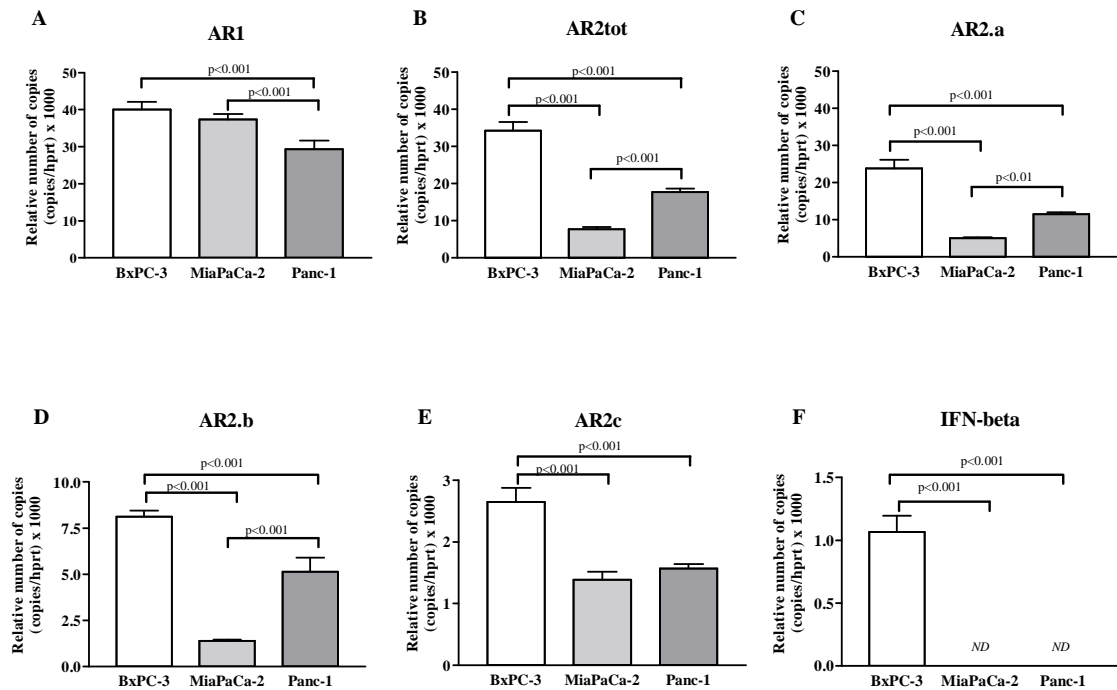


Figure 5A-F: Relative expression of type I IFN receptor (AR-1, AR-2 total, AR-2a, AR-2b, AR-2c) and IFN- β mRNA in human pancreatic cancer cell lines (BxPC-3, MiaPaCa-2, Panc-1), evaluated by quantitative RT-PCR. The amount of IFN receptor and IFN- β mRNA was calculated relative to the amount of HPRT mRNA and is given in arbitrary units. The soluble form of IFNAR-2a subunit was determined indirectly by subtracting IFNAR-2b and IFNAR-2c from IFNAR-2 total. Values represent the mean \pm SEM

Expression of type I IFN receptors and IFN- β mRNA

Since the susceptibility of cells to IFNs could reflect the different amount of corresponding receptors, we analyzed the expression of type I IFN receptors

(IFNAR-1 and IFNAR-2, short and long form) mRNA by real time quantitative RT-PCR in BxPC-3, MiaPaCa-2 and Panc-1 cell lines. Using sequence specific primers against the type I IFN receptor subunits, we detected the presence of IFNAR-1, IFNAR-2 total, IFNAR-2b and IFNAR-2c mRNA, normalized for the amount of the housekeeping gene HPRT. As shown in figure 5, the expression of IFNAR-1 mRNA was significantly higher in BxPC-3 and MiaPaCa-2 compared with Panc-1 (both $p<0.001$), while no difference was observed between BxPC-3 and MiaPaCa-2. In addition, the number of IFNAR-2a, IFNAR-2b and IFNAR-2c mRNA copies was higher in BxPC3 than in MiaPaCa-2 and Panc-1 ($p<0.001$).

Low expression of mRNA encoding for IFN- β was detected in BxPC-3, while it was undetectable in MiaPaCa-2 and Panc-1. We also investigated whether the potent IFN inducer, poly I:C, was able to stimulate pancreatic cancer cells to produce endogenous IFN- β . Treatment with poly I:C at the concentration of 100 $\mu\text{g/ml}$ strongly up-regulated the expression of IFN- β mRNA in only BxPC-3 (Fig. 6). The peak of IFN- β stimulation was detected after 2 hours of incubation with poly I:C, with an increase of about 80-fold compared to the control. After 24 hours the levels of IFN- β mRNA returned to the normality. In contrast, in the MiaPaCa-2 and Panc-1 cell lines, IFN- β mRNA was absent in normal baseline condition as well as after stimulation with poly I:C (100 $\mu\text{g/ml}$).

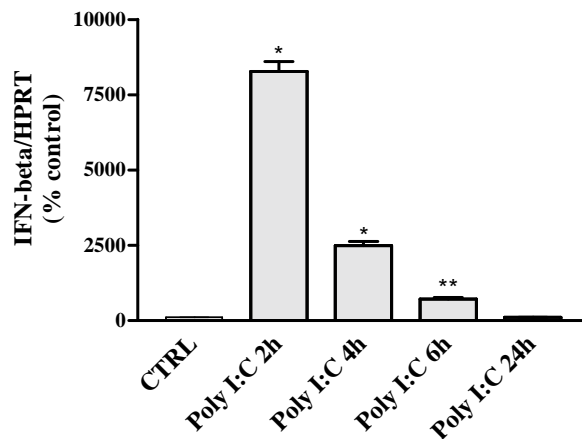


Figure 6: Relative expression of IFN- β mRNA after 2h, 4h, 6h and 24h of incubation with poly I:C (100 $\mu\text{g/ml}$) in BxPC-3 cell line, evaluated by quantitative RT-PCR. The amount of IFN- β mRNA was calculated relative to the amount of HPRT mRNA and is given in arbitrary units. Data (mean \pm SEM) are presented as percentage change over the control. *: $p<0.001$; **: $p<0.05$.

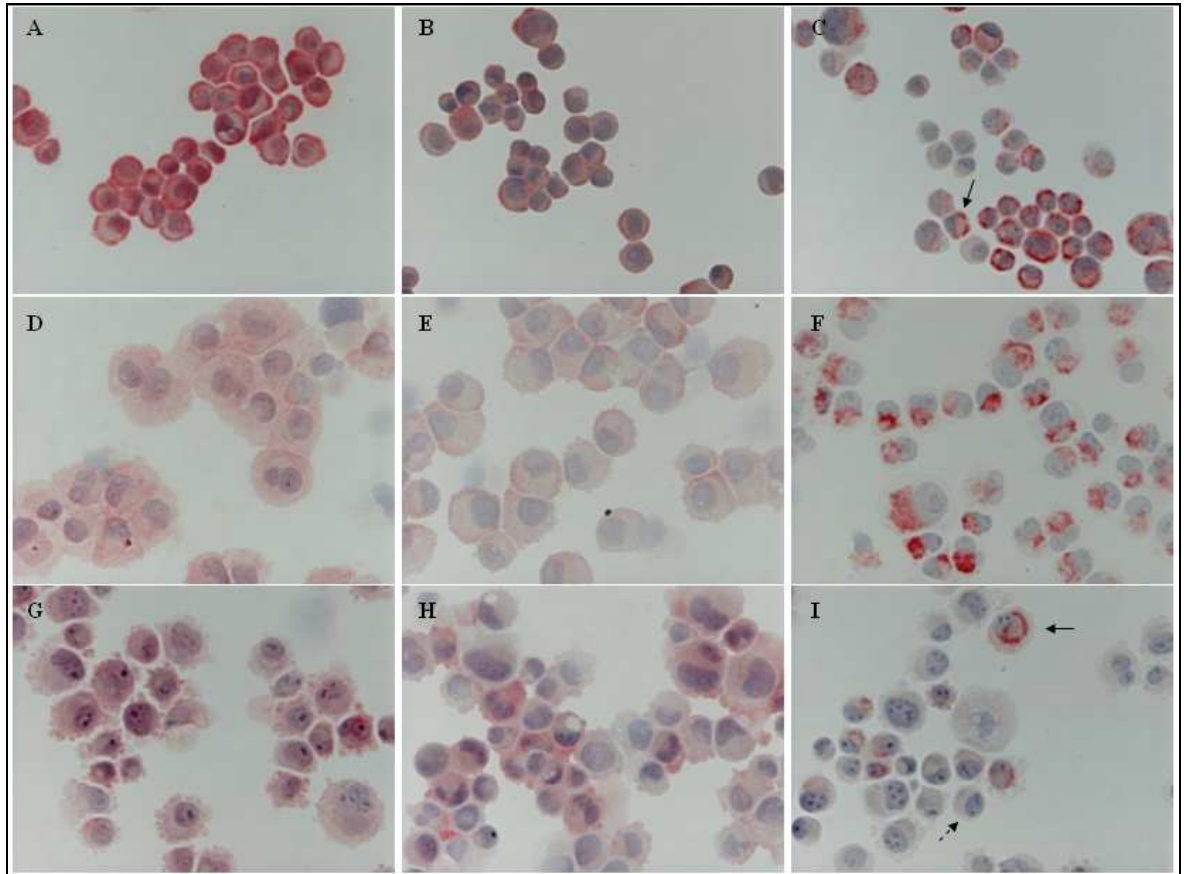


Figure 7: Immunocytochemical detection of IFNAR-1 (A,D,G), IFNAR-2b (B,E,H) and IFNAR-2c (C,F,I) receptors in BxPC-3 (A-C), MiaPaCa-2 (D-F) and Panc-1 (G-I). Magnification, x 400. The expressions of IFNAR-1 and IFNAR-2c are mainly membranous in BxPC-3 (A; C see solid arrow), while in Panc-1 both receptors are localized in the cytoplasm (G; I see solid arrow) and 60-70% of the cells are negative for IFNAR-2c (I, see dash arrow)

Immunocytochemistry

Specific immunoreactivity for IFN receptor subunits (IFNAR-1, IFNAR-2b and IFNAR-2c) was found in all 3 pancreatic cancer cell lines (Fig. 7A-I). It was strongly positive for IFNAR-1 in BxPC3 (Fig. 7A) and the staining was predominantly at the plasma membrane. On the other hand, MiaPaCa-2 (Fig. 7D) and in Panc-1 (Fig. 7G) the expression of IFNAR-1 was lower and particularly distributed in the cytoplasm. IFNAR-2b showed a comparable expression in BxPC-3 (Fig. 7B) and Panc-1 (Fig. 7H). In BxPC3 the immunostaining of IFNAR-2b was localized on the cytoplasm and on the membrane, while in Panc-1 the expression of IFNAR-2b is preferentially on the cytoplasm. In MiaPaCa-2 (Fig. 7E) the expression of this subunit was cytoplasmic and lower compared with the other 2 cell lines. IFNAR-2c is

mainly expressed on the plasma membrane and in the cytoplasm in BxPC-3 (Fig. 7C) and in MiaPaCa-2 (fig. 7F), respectively. In addition, in Panc-1 (Fig. 7I) this subunit is mainly expressed in cytoplasm, but the IFNAR-2c pattern is heterogeneous, in fact the staining is negative in about 60-70% of the cells.

Discussion

Although few trials criticized the intense toxicity of IFNs (21,22), several studies showed that combination of IFN- α with adjuvant chemoradiation therapy might increase response rates and achieve prolonged survival in patients with pancreatic cancer (4,23). In addition, the administration of IFN- α in combination with 13-cis retinoic acid or with 5-fluorouracil, leucovorin and cisplatin seems to have an increased antiproliferative effect in advanced pancreatic carcinoma (24-26). Interestingly, recent reports showed that regional adenovirus-mediated IFN- α gene transfer significantly suppressed the growth of subcutaneous xenografts of human pancreatic cancer cells in nude mice (27,28).

Whereas the role of IFN- α has been extensively studied, the effect of other type I IFNs on pancreatic cancer has been less evaluated. Preliminary reports suggested the possibility to use IFN- β in the treatment of pancreatic cancer (29-32). A high local production of IFN- β seems to have a strong antitumor effect on PANC02-H7, a highly metastatic mouse pancreatic carcinoma cell line successfully transfected with a vector containing a murine IFN- β gene. The IFN- β -secreting cells drastically lose their capacity to growth and metastasize in syngenic mouse, whereas the same cells transfected with a control vector remain highly tumorigenic and metastatic (29). A recent paper showed that the treatment of human pancreatic cancer cell lines with gemcitabine and human IFN- β gene entrapped in liposomes was more effective than each treatment alone (30). Busch *et al.* described the stabilization of the disease in a patient with incomplete resection of a pancreatic cancer, treated with IFN- β in combination with gemcitabine, cisplatin and radiotherapy (31). On the other hand, Recchia *et al.* showed few long-lasting responses and disease stabilization in patients with advanced pancreatic cancer by combining IFN- β with chemotherapy (5-fluorouracil, folinic acid, epirubicin, mitomycin C) and retinoids (32). However, the efficacy of IFN- β in the treatment of pancreatic cancer, the potential differences in antitumor activity with IFN- α and the involved mechanisms of

action are still poorly understood. Besides, in clinical practice one of the main limits of therapy with type I IFNs is the absence of molecular predictors, potentially useful in deciding whether a patient should be treated. This is a crucial point, considering that several tumours are completely or partially resistant even to high doses of IFNs. While, the loss of type I IFNs genes has been excluded to be a predictor factor of response to the treatment with IFN- α and IFN- β treatment (18), the role of the IFN receptor has not been reported so far.

Therefore, in the present study, we compared the anti-tumor effects of IFN- α and IFN- β , as well as the mechanisms that are involved in the growth inhibition of 3 human pancreatic cancer cell lines (BxPC-3, MiaPaCa-2 and Panc-1). Moreover, for the first time we evaluated the cellular distribution of type I IFN-receptor subtypes in these cell lines. We found that IFN- β potently inhibits cell proliferation already at very low concentrations (5-10 IU/ml), at least in BxPC3 and MiaPaCa-2. These concentrations can be achieved *in vivo*, since 12.3 IU/ml is the maximal IFN- β serum concentration reported in healthy subjects after subcutaneous administration of this cytokine (33,34).

The direct anti-tumor effects of IFN- α and - β are associated with the induction of apoptosis and cell cycle arrest. In BxPC-3 and MiaPaCa-2 both cytokines are able to induce apoptosis, but the increase in DNA fragmentation after IFN- β treatment occurred earlier and was considerably more potent than IFN- α . It is well known that type I IFNs induce apoptosis by activating the caspase cascade, releasing cytochrome-*c* from mitochondria, inducing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and promoting DNA fragmentation. However, this activation occurs late (after at least 48-72h) (35,36). Surprisingly, in BxPC-3 and MiaPaCa-2, a significant induction of apoptosis is already evident after 1 day of treatment with low dose IFN- β , while this effect is present only after 3 days of treatment with a moderate to high dose of IFN- α . Panc-1 results to be the most resistant cell line to both IFNs, showing a stimulation of apoptosis only at very high doses (IFN- α \geq 10000 IU/ml, IFN- β \geq 500 IU/ml). However, also in Panc-1 the induction of apoptosis is already observed after 1 day of incubation with IFN- β . In all 3 pancreatic cancer cell lines both IFNs induce a significant accumulation of cells in S phase compared to the control, suggestive of a cell cycle arrest in the late S phase. The block induced by IFN- β is more potent and earlier than that of IFN- α .

The differential expression and distribution of IFN receptor subunits could partially explain the different susceptibility of all 3 cell lines to IFN treatment. All type I IFNs activate a common receptor complex composed of two major

subunits, IFNAR-1 and IFNAR-2 (37,38). IFNAR-1 is considered the signaling subunit, as it is absolutely required for signal transduction. The IFNAR-2 is the subunit responsible for the interaction with the ligand. There are three forms of IFNAR-2, which are differentially spliced products of the same gene, e.g. the soluble (IFNAR-2a), short (IFNAR-2b) and long (IFNAR-2c) form (6, 39-41). The IFNAR-2c and IFNAR-1 subunits constitute the predominantly active form of the type I IFN receptor complex. IFNAR-2c is capable of binding ligand, but with a lower affinity (20-fold less) than the dimeric IFN receptor complex itself (42). Therefore, both receptor chains are required to form a high affinity-binding site and initiate signal transduction leading to the induction of IFN-responsive genes. The short form is able to bind type I IFNs, but does not couple to signal transduction because it lacks the signal transducing tail of IFNAR-2c (43). The soluble form may act as a regulator of free IFNs and, depending on concentration, lead to the neutralization or even enhancement of IFN bioactivity (44,45).

Quantitative RT-PCR study and immunocytochemical analysis demonstrate the presence of type I IFN receptor subunit transcripts and proteins in BxPC-3, MiaPaCa-2 and Panc-1 cells. The high expression of IFNAR-1 and IFNAR-2c subunits in BxPC-3 could explain the major sensitivity of this cell line to IFN treatment. In fact, as shown by Wagner *et al.*, increasing the cell surface levels of IFNAR2c in cancer cells enhances their sensitivity to the antiproliferative and apoptotic effects of type I IFNs (46). Besides, long-term cultures of IFNAR1-deficient mouse embryonic fibroblasts as well as IFN- β deficient cells result in the formation of transformed colonies, which could form tumors in nude mice (5). It is interesting to observe that, by immunocytochemistry we demonstrate striking differences in the subcellular localization and distribution of IFNs receptor subunits. While in BxPC-3 the staining for the active subunits of IFN receptor (IFNAR-1 and IFNAR-2c) are mainly membranous, in Panc-1 the expression of both subunits is preferentially in the cytoplasm. Besides, in Panc-1 about 60-70% of the cells exhibit no detectable levels of IFNAR-2c. This heterogeneity in IFNAR-2c expression may provide an additional explanation for the low sensitivity of these cells to IFN- α and IFN- β treatment. In Panc-1 it is possible a selection of cell type during type I IFNs treatment, with higher possibility to survive for IFNAR-2c negative cells. Therefore, all these data suggest that the high sensitivity of BxPC3 to IFNs treatment could be related to the strong expression of IFNAR-1 and IFNAR-2c and the main membranous localization. On the other hand, the low expression, cytoplasmic localization and heterogeneous staining of the active receptor in Panc-1 could explain the relative resistance of these cells to IFNs treatment. This is the first study, as

far as we know, showing the importance of expression, distribution and localization of type I IFNs receptor subtypes in the modulation of response to IFNs treatment in pancreatic cancer. Our data are also suggestive for a careful evaluation of IFNAR subtypes in pancreatic cancer before treatment with type I IFNs is considered.

Finally, the expression of IFN- β was investigated. BxPc-3 is the only cell line able to produce IFN- β mRNA in normal condition or after the stimulation with poly I:C, a synthetic double-stranded RNA copolymer of inosinic and cytidylic acids able to mimic a viral infection. It is noteworthy, that BxPC-3 has been described to be more differentiated than MiaPaCa-2 and Panc-1 (47). We cannot exclude a main role of the endogenous production of IFN- β in the differentiation of these cell lines, considering that IFNs are also differentiating factors (5,10).

Although IFN- α and IFN- β interact with the same receptor, the induction of a differential response can be explained by the diversity in the structure between both cytokines. There is only 35% sequence identity between both IFNs, and unlike IFN- α , IFN- β is glycosylated and includes a zinc ion (48,49). These differences generate different interactions and affinities for the related receptor. In fact IFN- β has a higher affinity (10-fold) than IFN- α (9). However, this cannot completely explain the difference in potency of cell growth inhibition between both cytokines, particularly in BxPC-3, where the IC₅₀ for IFN- β is 40 times lower than that of IFN- α . Differences in the interaction of these IFNs with their receptors might be also involved. In fact, although both IFNs induce tyrosine phosphorylation of the receptor subunits, IFN- β , but not IFN- α , induces the association of IFNAR-1 and IFNAR-2c chains, indicating that the specificity of signaling for distinct type I IFN subtypes is established by differential conformation of the receptor complex (43,50).

In conclusion, our study shows that IFN- β is significantly more effective than IFN- α in inducing cell growth inhibition in pancreatic cancer, because it induces a more potent and earlier cell cycle arrest and apoptosis activation compared with IFN- α . Considering that IFN- β stimulates apoptosis already at very low dose, this cytokine could be a more promising agent than IFN- α for the treatment of human pancreatic cancer, particularly in tumors with a high expression of IFNAR1 and IFNAR2c, supporting its use in future clinical investigation. In addition, there is clear *in vitro* evidence that differential expression levels and distribution of the IFNs receptor subunits play a role in the regulation of the response to type I IFNs therapy in pancreatic cancer. Future studies should investigate *in vivo* whether the intensity, subcellular

localization and distribution of IFNAR-1 and IFNAR-2c at immunohistochemistry might predict the response to therapy with IFN- β in pancreatic cancer.

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CHAPTER IV:

DIFFERENTIAL EFFECTS OF TYPE I INTERFERONS ON THE TUMOR GROWTH OF HUMAN ADRENAL CARCINOMA.



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Abstract

Adrenocortical carcinoma (ACC) is a rare tumor with a poor prognosis. Despite efforts to develop new therapeutic regimens, surgery remains the mainstay of treatment. In the present paper, we evaluated the anti-tumor effects of type I interferons (IFN- α and IFN- β) on human ACC cell lines (H295 and SW13).

As determined by quantitative RT-PCR analysis and immunocytochemistry, H295 and SW13 cells expressed the active type I IFN receptor mRNA and protein (IFNAR-1 and IFNAR-2c subunits). Both IFNs significantly inhibited ACC cell growth in a dose-dependent manner, but the effect of IFN- β 1a (IC₅₀: 5 IU/ml, maximal inhibition: 96% in H295; IC₅₀: 18 IU/ml, maximal inhibition: 85% in SW13) was significantly more potent than that of IFN- α (IC₅₀: 57 IU/ml, maximal inhibition: 35% in H295; IC₅₀: 221 IU/ml, maximal inhibition: 60% in SW13). Whereas in H295 cells both IFNs induced apoptosis and accumulation of the cells in S phase, in SW13 the anti-tumor mechanism is modulated by the cell cycle arrest only. Interestingly, only IFN- β potently suppressed IGF-2 mRNA expression in H295.

In conclusion, IFN- β is much more potent, compared to IFN- α , in its inhibitory effect on ACC cell proliferation *in vitro* via the induction of apoptosis and cell cycle arrest. Further studies are required to establish whether IFN- β has comparable potent tumor growth inhibitory effects *in vivo*.

Introduction

Adrenocortical carcinoma (ACC) is a rare tumor with a dismal prognosis. The incidence of AAC in the population is 0.5 to 2 per million and about two-third of ACC are functional, producing cortisol, aldosterone, androgens, or estrogens (1, 2).

Complete surgical resection is currently the only potentially curative therapy for localized adrenal cortical carcinoma (3). When complete resection is not possible, or in advanced disease, the treatment of choice is chemotherapy with mitotane (2). Mitotane is an adrenolytic compound with a specific activity on the adrenal cortex (4). The efficacy of the treatment is approximately 30%, dependent of the stage of the tumor. This drug gives several side effects in central nervous system, liver, kidney, and bone marrow and long-term therapy is indicated only in case of a clinical response (2). In addition, there are no conclusive data showing favourable effects on survival and quality of life after the treatment with mitotane alone or in combination with chemotherapy in ACC (5). Therefore, novel treatment strategies are clearly required for this carcinoma.

In vitro and *in vivo* studies have shown the efficacy of type I interferons (IFNs) in the treatment of several tumors, alone or in combination with chemotherapy (6, 7). Type-I IFNs primarily include IFNs- α , IFN- β and IFN- ω , that interact with same receptor complex, composed by two subunits: IFNAR-1 and IFNAR-2 (8, 9). These cytokines modulate anti-tumor activity through different mechanisms, e.g. cell cycle arrest, induction of apoptosis, activation of NK cells, T cells, monocytes and macrophages, induction of surface antigen expression on tumor cells, as well as inhibition of angiogenesis and the production of various growth factors (10). It has been described that type I IFNs are able to downregulate the expression of gene and protein release of insulin-like growth factor-2 (IGF-II) in some cancers (11, 12). Considering that, IGF-II is highly expressed by more than 90% of the ACC and being involved in adrenal growth and tumorigenesis of this cancer (13), type-I IFNs could be of potential interest in the treatment of ACC.

To further explore the possibilities of new medical therapies in ACC, we investigated the *in vitro* effects and the mechanism of action of type-I IFNs (IFN- α 2b and IFN- β) on the growth of two established human ACC cell lines (H295, SW13).

Material and method

Cell lines and culture conditions

Human H295R cells, a human adrenocortical carcinoma cell line, were obtained from ATCC (Manassas (VA), United States). As previously shown, these cells produce several corticosteroid hormones (14). SW13 adrenal tumor cells were obtained from ECACC (Salisbury, Wiltshire, UK).

The cells were cultured in a humidified incubator containing 5% CO₂ at 37°C in 75 cm² culture flasks (Corning Costar, Amsterdam, The Netherlands). The culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12K medium, supplemented with 5% FCS, penicillin (1x10⁵ U/l), fungizone (0.5 mg/l) and L-glutamine (2 mmol/l). Periodically, cells were confirmed as *Mycoplasma*-free. Cells were harvested with trypsin EDTA 10% and resuspended in culture medium. Before plating, cells were counted microscopically using a hemocytometer. Trypan Blue staining was used to assess cell viability, and always exceeded 95%. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

Drugs and Reagents

Human recombinant IFN- α -2b (Roferon-A) was purchased from Roche® (Almere, The Netherlands). Human recombinant IFN- β -1a was purchased from Serono Inc. (Rebif, Rockland, MA). Human recombinant IFN- β -1b was obtained from Schering (Mijdrecht, The Netherlands). Anti-human IFN- β neutralizing antibody was purchased from Sigma-Aldrich (St. Louis, MA). Caspase inhibitors Devd-cho, Lehd-cho and Ietd-cho were purchased by Biosource (Brussel, Belgium). Mitotane ((1,1-dichloro-2(o-chlorophenyl)-2(p-chlorophenyl)ethane), Lysodren) was obtained from Bristol-Myers Squibb (Sermoneta, Italy). All compounds were stored at -20°C, and the stock solution was diluted according to the manufacturer instructions.

Quantitative RT-PCR

Quantitative PCR was performed as described previously (15, 16). Briefly, for the detection of interferon receptors (IFNAR1, IFNAR2c, IFNAR2b) and IGF-II total RNA was isolated using a commercially available kit (Roche®, Almere, The Netherlands) according to the manufacture procedure.

cDNA was synthesized using 10 μ l (10 μ g/ μ l) RNA, in a Tris buffer (50 mmol/L Tris-HCL (pH 8.3), 100 mmol/L KCL, 4 mmol/L DTT, and 10 mmol/L MGCL₂), together with 5 ng Oligo(16)12-18 Primer (InVitrogen, Breda, The Netherlands), 1 mmol/L of each deoxynucleotide triphosphate, 10 units RNase inhibitor, and 2 units avian myeloblastosis virus Super Reverse Transcriptase (HT biotechnology) in a final volume of 40 μ L. This mixture was incubated by 65°C for 10 min followed by 42°C for 60 min., (biometra, Westburg, The Netherlands).

A quantitative PCR was performed using the TaqMan® Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI PRISM® 7700 sequence detection System (Perkin Elmer Applied Biosystems, Groningen, The Netherlands) for real-time amplifications, according to manufacturer's protocol. The assay was performed using 12.5 μ l TaqMan® Universal PCR Master Mix (Applied Biosystems, The Netherlands), 2.5 μ l primer mix contain: forward primer (500 nmol for IFNAR-2c; 300 nmol for IFNAR-1, IFNAR-2 total, IFNAR-2b and IGF-II), reverse primer (500 nmol for IFNAR-2c; 300 nmol IFNAR-1, IFNAR-2total, IFNAR-2b and IGF-II), probe (100 nmol for IFNAR-1 IFNAR-2total, and IGF-II; 200 nmol IFNAR-2b and IFNAR-2c) and 10 μ l cDNA template. After an initial heating at 95°C for 8 min, samples were subjected to 40 cycles of denaturation at 95°C for 15 sec and annealing for 1 min at 60°C. The primer and probe sequences were purchased from Biosource (Brussel, Belgium). The sequence of the primers IFNAR-1, IFNAR-2 total, IFNAR-2b and IFNAR-2c and IGF-2 were described in table 1 (17).

Table 1: Primers and probes used for the quantitative RT-PCR.

IFNAR-1	
Forward	5'-CCCAGTGTGTCTTTCCTCAAA-3'
Reverse	5'-AAGACTGGAGGAAGTAGGAAAGC-3'
Probe	5'-FAM-TCCGCGTACAAGCATCTGATGGA-TAMRA-3'
IFNAR-2 (total form)	
Forward	5'-AGTCAGAGGGAATTGTTAAGAAGCA-3'
Reverse	5'-TTTGGGAATTAACCTGTCAATGATATAGGTG-3'
Probe	5'-FAM-AAACCCGAAATAAAAG GAAACATGAGTGGAATT-TAMRA-3'
IFNAR-2c (long form)	
Forward	5'-TGACAAGCACCATAGTGACACTGA-3'
Reverse	5'-TAGGAAATGGCCAGGCTAAAAA-3'
Probe	5'-TGGATTGGTTATATATGCTTAAG AAATAGCCTCCCCA-TAMRA-3'
IFNAR-2b (short form)	
Forward	5'-GCTTAAGAAATAGCCTCCCCAAA-3'
Reverse	5'-CTGTGAATAGCCACTGCATTCC-3'
Probe	5'-TCTTGAGGCAAGGTCTCGCTAAGGGC-TAMRA-3'
IGF-2	
Forward	5'-CCAAGTCCGAGAGGGACGT-3'
Reverse	5'-TTGGAAGAACTTGCCACG-3'
Probe	5'-FAM-ACCGTGCTTCCGGACAACTTCCC-TAMRA-3'

The estimated copy numbers were obtained according to the method described by Swillens et al. (18). IFNAR-1, IFNAR-2 total, IFNAR-b, IFNAR-2c and IGF-II were normalised by the amount of RNA. Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the RNA the cDNA reactions were also performed without reverse transcriptase and amplified with each primer-pair. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of cDNA template in parallel with cDNA samples. As a positive control for the PCR reactions of type I IFN receptors and IGF-2 human DNA was amplified in parallel with the cDNA samples.

Immunocytochemistry

Cytospins preparations of H295 and SW13 cells were fixed with acetone (10 min.) and incubated for 30 minutes at room temperature with antibodies to human IFNAR-1 (rabbit polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) and human IFNAR-2c (monoclonal antibody, Dr. E. Croze, Berlex Biosciences, Richmond, CA).

Finally, a peroxidase complex (IL Immunologic, Duiven, The Netherlands) for IFNAR-1 or a standard streptavidin-biotinylated alkaline phosphatase detection system (IL Immunologic, Duiven, The Netherlands) for IFNAR-2c were used according to the manufacturer's recommendations to visualize the bound antibodies.

Negative controls for the immunohistochemistry included omission of the primary antibody.

Cell proliferation assay

After trypsinization, the cells were plated in 1 ml of medium in 24-well plates at a density of 10^5 cells/well for H295 and of 5×10^3 SW13. The plates were then placed in a 37°C , 5% CO_2 incubator. Two days later the cell culture medium was replaced with 1ml/well medium containing various concentrations of drugs (IFN- α : 0-1000 IU/ml; IFN- β 1a: 0-100 IU/ml; IFN- β 1b: 0-100 IU/ml). Quadruplicates of each treatment were performed. Plates were further incubated at 37°C and 5% CO_2 . After 3 and 6 days of treatment the cells were harvested for DNA measurement. Plates for 6 days were refreshed after 3 days and compounds were added again. Measurement of total DNA-contents, representative for the number of cells, was performed using the bisbenzimidazole fluorescent dye (HoechstTM 33258, Boehringer Diagnostics, La Jolla, CA), as previously described (19)

To evaluate the specificity of the effects of IFN- β 1a on cell growth, the cells were exposed to IFN- β 1a with an anti-human IFN- β neutralizing antibody. H295 cells (10^5 cells/well) were plated in 24-well plates and after 2 days of incubation the medium containing IFN- β 1a (5 IU/ml), in the absence or in presence of anti-human IFN- β neutralizing antibody (4 $\mu\text{g/ml}$), was added. After 3 days the plates were collected for DNA measurement, as previously described. IFN- β 1a alone or in combination with anti-human IFN- β neutralizing antibody, have been incubated for overnight at 4°C prior to be added to H295 cells, in order to antagonize the IFN- β activity.

Apoptosis assay

Apoptosis was evaluated by the analysis of the DNA fragmentation. After plating of 10^5 cells/well for H295 and 2×10^4 cells/well for SW13 on 24 well plates, cells were incubated at 37°C . After two days the cell culture medium was replaced with 1ml/well medium containing various concentrations of drugs (IFN- α : 0-1000 IU/ml; IFN- β 1a: 0-100 IU/ml; IFN- β 1b: 0-100 IU/ml). Quadruplicates of each treatment were performed. After 1 day of incubation, apoptosis was assessed using a commercially available ELISA kit (Cell Death Detection ELISA^{Plus}, Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used, as previously described (20). Apoptosis was expressed as percentage to control.

To evaluate the role of caspases involved in the induction of apoptosis after IFN- β 1a treatment, we studied the effects of caspase inhibitors in combination with IFN- β 1a in H295 cells. Cells (10^5 cells/well) were plated in 24-well plates and after 1 day of incubation, 1ml of the medium containing 10 μM of different caspase inhibitors: Devd-cho (blocks caspase 3), Ietd-cho (blocks caspase 8), Lehd-cho (blocks caspase 9) was added. After 1 day of incubation, IFN- β 1a (5 IU/ml) in the absence or in the presence of the caspase inhibitors, was incubated for 24 hours. Plates were collected for DNA fragmentation detection, as previously described.

Cell cycle analysis

Cells 2×10^5 for H295 and 5×10^5 for SW13 were plated on 12 well plates. After 1 day the medium was changed with fresh medium (control) or with fresh medium plus IFN- α 2b (500 and 1000 IU/ml) or IFN- β 1a (50 and 100 IU/ml). After 3 days of incubation (confluency of about 60-70%), cells were harvested by gentle trypsinization, washed with cold phosphate-buffered calcium and magnesium free saline (PBS), and collected by centrifugation. Cells were re-suspended in 200 μl of PBS and fixed in 70% ice-cold ethanol with an overnight incubation at -20°C . After brief centrifugation, cells were washed once with PBS and incubated for 30 minutes at 37°C in PBS containing 40 $\mu\text{g/ml}$ of propidium iodide (Sigma Aldrich, Zwijndrecht, The Netherlands) and 10 $\mu\text{g/ml}$ of DNase-free RNase (Sigma Aldrich, Zwijndrecht, The Netherlands). For each tube 20000 cells were immediately measured on a

FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) and analyzed using CellQuest Pro Software.

Cortisol determination

After trypsinization, the H295 cells were cultured in 24-well plates at a density of 10^5 cells/well. The plates were then placed in a 37°C, 5% CO₂ incubator. After two days later the cell culture medium was replaced with 1ml/well medium containing increasing various concentrations of drugs (IFN- α : 0-1000 IU/ml; IFN- β 1a: 0-100 IU/ml). Medium were refreshed after 3 days and compounds were added again. On day 6, medium was removed and fresh medium and drugs with forskolin 10 μ M was added to the cells for 24 hours. Forskolin stimulates the adenylate cyclase system. In presence of forskolin, cortisol is the major protein secreted by the adrenal carcinoma cell line H295 in contrast to other steroids protein (14). After 24 hours the media were collected and stored at -20°C until the measurement of cortisol.

Human cortisol concentrations were determined by a non-isotopic, automatic chemiluminescence immunoassay system (Immulate, DPC Inc., Breda, The Netherlands) according to the manufacturer procedures. Intra- and interassay CV's were 5.6% and 7.8%, respectively.

Statistical analysis

All experiments were carried out at least three times and gave comparable results. For statistical analysis GraphPad Prism™ 3.0 (GraphPad Software, San Diego, USA) was used. Relative 50% growth-inhibitory concentrations (IC₅₀) and maximal inhibitory effect were calculated using nonlinear regression curve-fitting program. The comparative statistical evaluation among groups was firstly performed by ANOVA test. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. The unpaired Student t-test was chosen to analyze the differences in concentration-effect curves (IC₅₀ and maximal inhibitory effect) and effects in cell cycle modulation between different types or preparations of IFNs, as well as the differences of the growth inhibitory effects of IFNs after 3 and 6 days of treatment. Correlation analyses were performed using Pearson's coefficients. In all analyses, values of $p < 0.05$ were considered statistically significant. Data are reported as mean \pm SEM.

Results

Expression of type I IFN receptor mRNA and proteins in H295 and SW13 cells

The activity of type I IFNs is modulated by a common receptor. We generated a set of specific primers to analyze the expression of IFNAR-1 and IFNAR-2 (total, short and long form) mRNA in H295 and SW13 cells by quantitative RT-PCR. In both cell lines we detected the presence of IFNAR-1, IFNAR-2 total, IFNAR-2b and IFNAR-2c (fig. 1). IFNAR-1 mRNA expression, normalized for the amount of the total RNA, was higher than IFNAR-2 total in both cell lines. Among the IFNAR-2 subunits, IFNAR-2c was the form expressed at the relatively lowest level.

Specific immunoreactivity for the two active IFN receptor subunits (IFNAR-1 and IFNAR-2c) was found in H295 and SW13 cells at immunocytochemistry

(fig. 2). It was positive for IFNAR-1 mainly localized on the membrane (fig.2 A,C) and strongly positive for IFNAR-2c on the membrane and cytoplasm (fig.2 B,D).

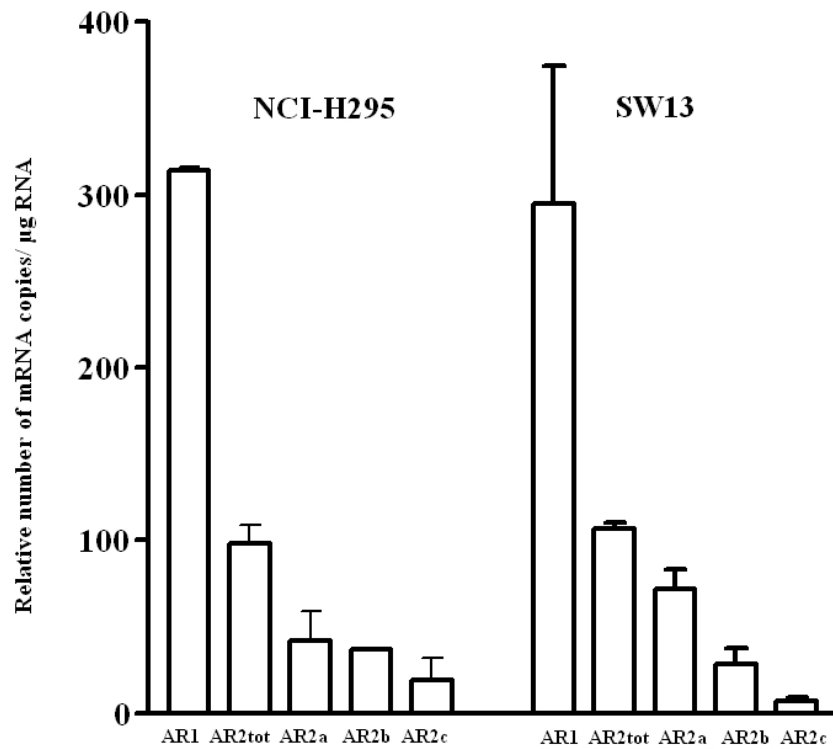


Figure 1: Relative expression of type I interferon receptor (AR-1, AR-2 total, AR-2a, AR-2b, AR-2c) mRNA in the human adrenal cancer cell lines H295 and SW13, evaluated by quantitative RT-PCR. The amount of relative copies of IFN receptor RNA was calculated correcting for the amount of total RNA. The soluble form of IFNAR-2a subunit was determined indirectly by subtracting IFNAR-2b and IFNAR-2c from IFNAR-2 total. Values represent the mean \pm SEM.

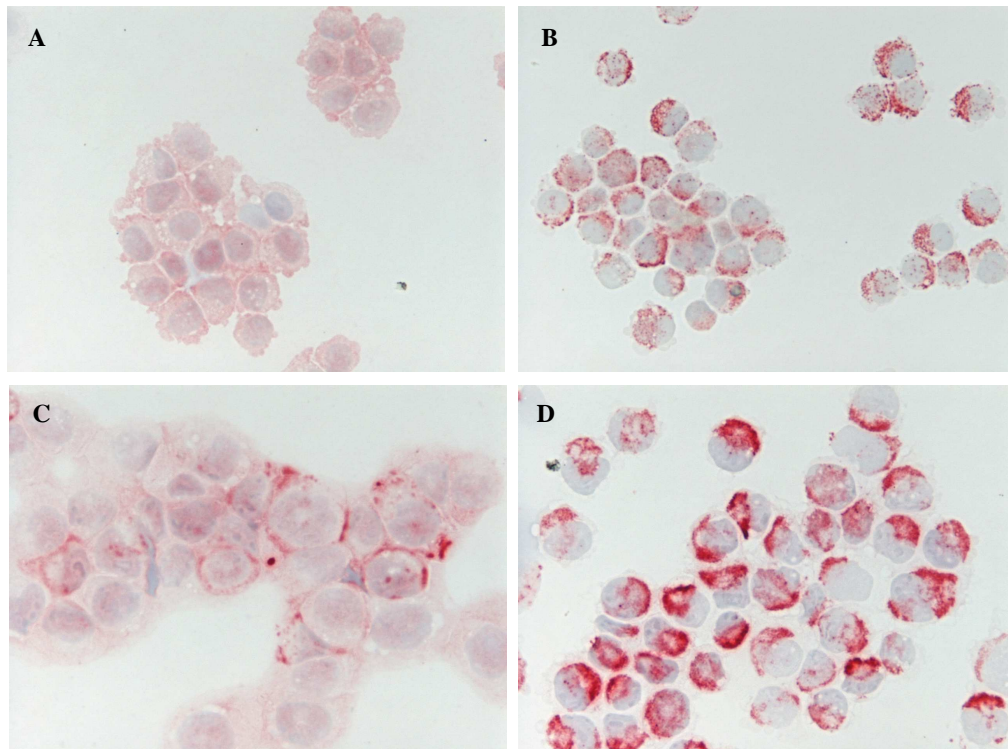


Figure 2: Immunocytochemical detection of the functional IFN receptor subunits IFNAR-1 (A, C) and IFNAR-2c (B, D) in human adrenal cancer H295 (A, C) and SW13 (B, D) cell lines. A strong IFNAR-1 and IFNAR-2c immunostaining is evident mainly in the membrane and lesser in the cytoplasm. Magnification, x 400.

Effects of two different types of IFN- β on cell growth and apoptosis

We evaluated the effect of two commercially available INF- β preparations on cell proliferation and apoptotic activity in H295 cells. IFN- β 1a (Serono, amino acid sequence and glycosylation equal as native IFN- β) and IFN- β 1b (Schering, one amino acid change and absence of glycosylation compared to the native IFN- β).

The effect of IFN- β 1a on cell proliferation was significantly more potent compared with IFN- β 1b after 6 days of treatment (IC_{50} : 9.7 ± 2.7 IU/ml vs. 87.0 ± 3.8 IU/ml, $p < 0.01$; maximal inhibition effect on cell proliferation $88 \pm 7\%$ vs. $51 \pm 10\%$, respectively for IFN- β 1a and IFN- β 1b, $P < 0.01$) (Fig.3 A).

In H295 cells both types of INF- β preparations increased DNA fragmentation in a dose-dependent manner, but in concordance with the effect of cell

proliferation, IFN- β 1a was more potent than IFN- β 1b (IFN- β 1a, EC_{50} : 5.0 ± 1.4 IU/ml, vs. IFN- β 1b, EC_{50} : 249 ± 2 IU/ml $p < 0.01$) (Fig.3 B).

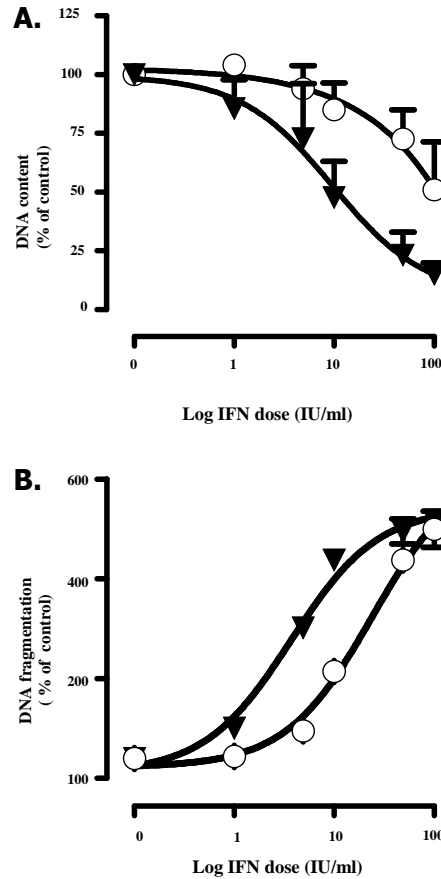


Figure 3: Dose-dependent effect of two different IFN- β preparations on cell growth expressed as DNA content (A) after 6 days of incubation, and on apoptosis, expressed as DNA fragmentation (B), after 24 hours of incubation, on H295 human adrenal carcinoma cells.

Data are expressed as percentage of control and represent the mean \pm SEM. Control is set as 100 percent.

▼: IFN- β 1a
○: IFN- β 1b

Effects of type I IFNs, IFN- α 2b and IFN- β 1a, on cell proliferation

After 6 days of incubation, IFN- α 2b and IFN- β 1a significantly suppressed the growth of H295 and SW13 cells in a dose-dependent manner (Fig.4 A,B). H295 was significantly more sensitive than SW13, as shown by the differences in IC_{50} after 6 days of treatment with IFN- α 2b (57.0 ± 2.3 IU/ml and 221.1 ± 1.3 IU/ml, H295 and SW13, respectively; $p < 0.01$) and IFN- β 1a (5.4 ± 1.3 IU/ml and 18.1 ± 1.3 IU/ml, H295 and SW13, respectively; $p < 0.01$). There was no statistically significant difference between the IC_{50} values of 3 and 6 days for every IFN. After 6 days of incubation IFN- β 1a induced in both cell lines a statistically significant cell growth inhibition already at very low concentrations (1 IU/ml, $P < 0.01$, vs. control).

The growth-inhibitory effect of IFN- β 1a was significantly more potent than that of IFN- α 2b, as shown by the higher maximal inhibition of proliferation induced by IFN- β 1a, compared with IFN- α 2b (H295: $96 \pm 7\%$ and $35 \pm 2\%$, respectively, $p < 0.01$; SW13: $85 \pm 7\%$ and $60 \pm 3\%$, respectively, $p < 0.01$) after 6 days of treatment, as well as by the lower IC_{50} of IFN- β 1a compared with IFN- α 2b ($p < 0.01$). In addition, the concentration used to obtain the maximal inhibition of proliferation is 100 IU/ml for IFN- β 1a and 10 times higher (1000 IU/ml) for IFN- α 2b. Comparable results were observed by a thymidine incorporation assay (data not shown).

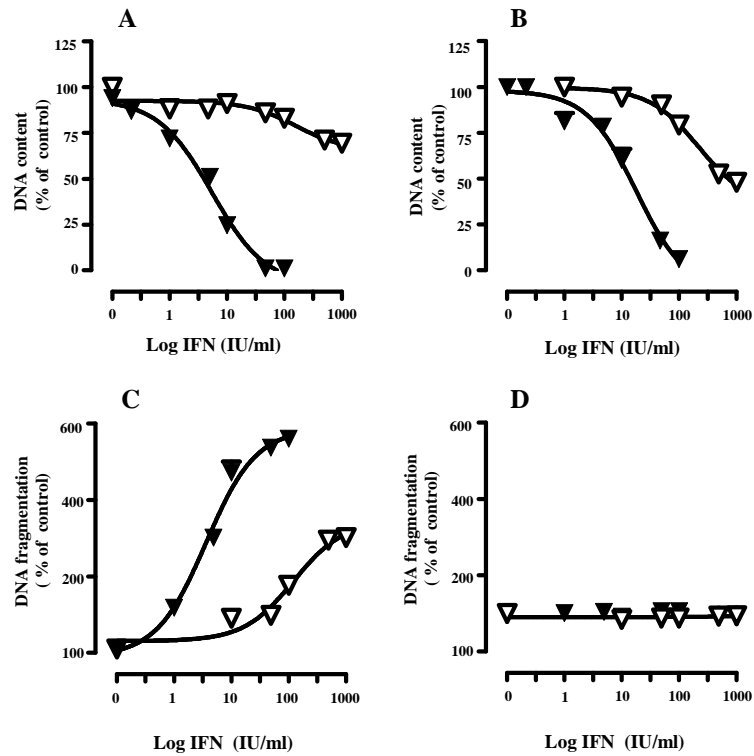


Figure 4: Doses-dependent effect of interferon treatment on cell proliferation, expressed as DNA content (A, B), after 6 days of incubation, and on apoptosis, expressed as DNA fragmentation (C, D), after 24 hours of incubation, in human adrenal cancer H295 cells (A, C) and on SW13 cells (B, D). The mean DNA contents in control were: 7257 ± 245 ng/well for H295 and 6870 ± 326 ng/well for SW13. Data are expressed as percentage of control and represent mean \pm SEM. Control is set as 100 percent.

▼: IFN- β 1a
 ▽: IFN- α 2b

To clarify the specificity of action of IFN- β 1a we evaluated the effects on cell growth after a pre-absorption of IFN- β 1a with neutralizing antibodies against IFN- β in H295 cells. The anti-tumor activity of IFN- β 1a (5 IU/ml) was completely abolished by immuno-neutralization (fig.5). In fact, no significant difference has been observed between DNA contents of the control vs IFN- β 1a + neutralizing antibodies against IFN- β or neutralizing antibodies alone, while IFN- β 1a alone induced a significant decrease in DNA contents compared to the control ($p < 0.01$).

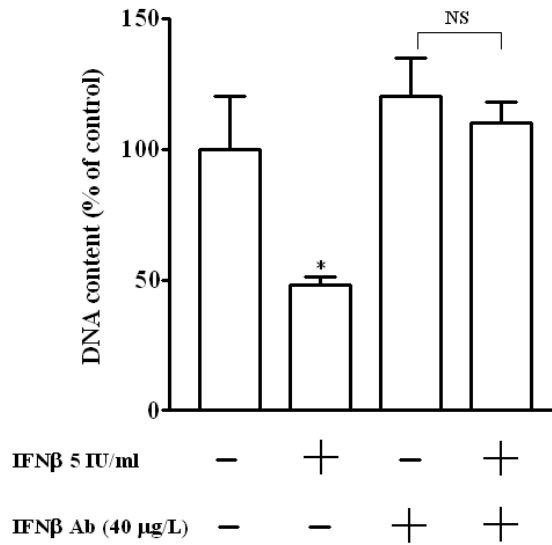


Figure 5: Effects of IFN- β (5 IU/ml) on cell growth after 72 hours of incubation, alone and following overnight pre-absorption with neutralizing antibodies against IFN- β (IFN-beta Ab) at the dose of 40 μ g/l All data are expressed as percentage of control. *: $p < 0.01$ vs. control.

Effects of type-1 IFNs, IFN- α 2b and IFN- β 1a, on apoptosis

After 1 day of treatment with IFN- β 1a and IFN- α 2b we measured the DNA fragmentation to investigate the effect on apoptosis (fig.4 C,D). In H295, a striking dose-dependent induction of apoptosis was observed after IFN- β 1a treatment (EC_{50} : 5.0 ± 1.4 IU/ml). The maximal increase of DNA fragmentation induced by 100 IU/ml of IFN- β 1a was about 6 times higher compared with untreated control cells. IFN- β 1a stimulated apoptosis in H295 cells already at very low concentrations (1 IU/ml, $p < 0.01$). A less effective dose-dependent induction of apoptosis was observed following IFN- α 2b exposure of H295 cells (EC_{50} : 249 ± 2 IU/ml). The maximal increase of DNA

fragmentation was about 2.5 times compared to the control after 1000 IU/ml of IFN- α 2b. Interestingly, no effect on apoptosis was observed after treatment with IFN- α 2b and IFN- β 1a in SW13.

To study the role of caspases 3, 8 and 9 in the induction of apoptosis by IFN- β 1a in H295 cells we tested if specific caspase inhibitors were able to block the IFN- β -induced DNA fragmentation. As shown in figure 6 the stimulation of apoptosis by IFN- β 1a is completely blocked by Devd-cho, a specific inhibitor of caspase-3, while it is partially counteracted by the use of specific inhibitors for caspase-8 (Ietd-cho) and -9 (Lehd-cho).

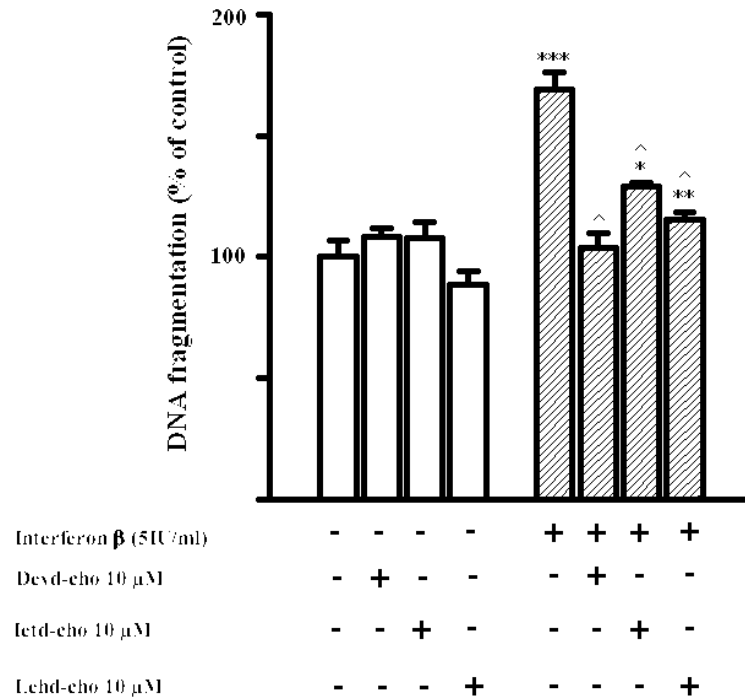


Figure 6: Effect of specific caspase inhibition on apoptosis induced by IFN- β in H295 cells. After 24 hours of preincubation with 10 μ M of Devd-cho (caspase 3 inhibitor), Ietd-cho (caspase 8 inhibitor), Lehd-cho (caspase 9 inhibitor), the cells were incubated for further 24 hours with 5 IU/ml IFN- β in the presence or absence of the caspase inhibitors. Apoptose is expressed as DNA fragmentation. Values are expressed as the percentage of control (untreated cells). +: compound present. *: P<0.05 vs. relative control group; **: P<0.01 vs. relative control group, ***: P<0.001 vs. relative control group; ^: P<0.01 vs. IFN- β without caspase inhibitors

Effects of type-I IFNs, IFN- α 2b and IFN- β 1a, on cell cycle

We evaluated the effect of treatment with IFN- α 2b (1000 IU/ml and 500 IU/ml) and IFN- β 1a (100 IU/ml and 10 IU/ml) on cell cycle distribution after 3 days of incubation (Table 2).

In the H295 and SW13 cells both IFNs induced a significant and dose dependent accumulation of cells in the S-Phase (IFN- α 2b 1000 IU/ml and IFN- β 1a 100 IU/ml: $p < 0.001$ vs control in both cells; IFN- α 2b 500 IU/ml and IFN- β 1a 50 IU/ml: $p < 0.05$ vs control in H295 and $p < 0.01$ vs control in SW13 cells), while no significant change in G₁/G₀ distribution was observed (table 2). The accumulation of cells in S-phase after treatment with both IFNs was significantly higher in the SW13 compared with H295 cells. In addition, the proportion of cells in G₂/M phase decreased significantly in comparison with the control in both cells lines. These data suggested that adrenal cancer cells exhibited a prolonged stay in S phase failing to transit into G₂/M phases after treatment with type I IFNs.

Finally, the cell cycle analysis revealed an increase in the percentage of H295 cells with subdiploid DNA content (sub-G₀ phase) after IFN- β 1a (100 IU/ml: $p < 0.001$; 50 IU/ml: $p < 0.05$) and high dose of IFN- α 2b (1000 IU/ml: $p < 0.05$), confirming the induction of apoptosis mainly after IFN- β 1a, as previously shown by the DNA fragmentation analyses.

Biochemical effects

The effect of both IFNs on forskolin induced cortisol production in H295 cells is comparable to the relative cell proliferation inhibition. In fact, we did not observe any significant difference between IC₅₀ of cortisol (IFN- β 1a: 8.4 ± 0.2 pmol/l; IFN- α 2b: 450 ± 390 pmol/l) and cell proliferation. This suggests that there is no effect on cortisol secretion and that the decrease in cortisol production is related to the decrease in the number of cells.

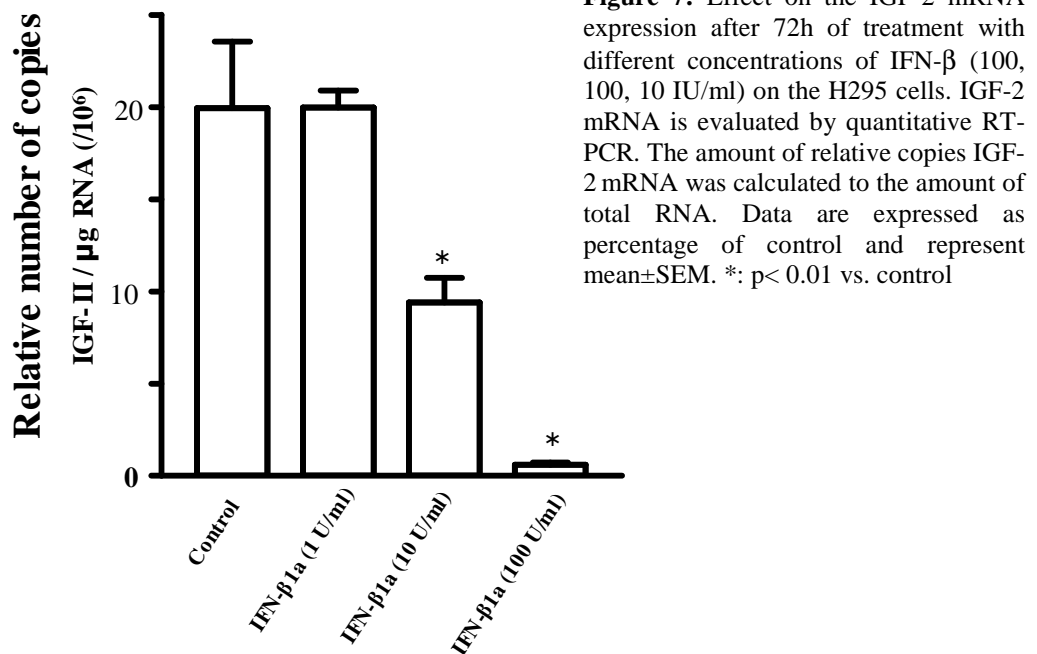
Table 2: Cell cycle distribution H295 and SW13 human adrenal carcinoma cells.

Cell line	G ₁ /G ₀		S-Phase		G ₂ /M-Phase		Sub- G ₀	
	H295	SW13	H295	SW13	H295	SW13	H295	SW13
Treatment								
<i>Control</i>	61.9±1.5	64.3±0.1	13.1±0.4	15.6±1.4	23.8±0.4	17.1±1.0	1.4±0.1	0.3±0.1
<i>IFN-α2b 1000 IU/ml</i>	58.1±1.3	60.5±1.8	20.0±0.7 **	27.25±0.6 **	19.1±0.1 #	12.6±0.3 #	3.5±0.1 #	0.3±0.1
<i>IFN-α2b 500 IU/ml</i>	62.5±1.2	60.2±1.7	14.8±0.1 #	22.8±0.6 *	19.7±1.2 #	14.4±1.8	2.3±0.6	0.4±0.1
<i>IFN-β1a 100 IU/ml</i>	59.4±4.0	59.1±2.4	23.9±.05 **	27.7±1.1 **	15.7±0.5 *	12.6±0.8 #	6.0±0.1 **	0.3±0.1
<i>IFN-β1a 50 IU/ml</i>	58.8±2.9	60.4±0.4	15.0±0.1 #	22.3±0.6 *	21.6±0.4 #	13.6±0.1 #	3.0±0.1 #	0.2±0.1

Effect of type-1 IFNs, IFN- α 2b and IFN- β 1a, on IGF-II transcription

The expression of IGF-II mRNA was detectable in H295 cells ($20 \pm 3 \times 10^6$ copies mRNA IGF-II/ μ g RNA), whereas it was undetectable in SW-13.

In H295 cells the transcription of IGF-II gene seems to be modulated by the incubation with IFN- β 1a. We observed a dose-dependent decrease in the number of copies of IGF-II mRNA after 3 days of treatment with IFN- β 1a (fig 7). In contrast, IFN- α 2b was unable to modify the expression of IGF-II gene in H295 cells (data not shown).



Discussion

Type I IFNs, initially identified for their ability to modulate antiviral response of cells, are also implicated in cell differentiation, cell growth control, immune response, and play an important role in the anti-tumor defense (6,7,11,12). These cytokines modulate a variety of biological response after

activation of a common receptor complex composed by two subunits: IFNAR-1 and IFNAR-2. IFNAR-1 is considered the signal subunit chain. There are three forms of IFNAR-2, which are differentially spliced products of the same gene. IFNAR-2a, soluble form, acts as a regulator of the free IFNs (21, 22). IFNAR-2b, short form, is able to bind type-1 IFN but does not couple to signal transduction because it lacks the signal transduction tail (23); IFNAR-2c, long form, characterized by its long cytoplasm tail, constitutes together with the IFNAR-1 subunit the predominant active form of the type-1 receptor complex. IFNAR-2c is capable to bind type I IFNs, but with a lower affinity (20 fold less) than the dimeric IFN receptor complex (24).

In the present study, we evaluated the anti-tumor effects of type I IFNs (IFN- α 2b, IFN- β 1a) in the human adrenal cancer H295 and SW13 cells. The expression of mRNA and protein for the active subunits of type I IFN receptor (IFNAR-1 and IFNAR-2c) was detected by quantitative RT-PCR and immunocytochemistry, respectively, in H295 and SW13 cells. Both IFNs showed a dose dependent inhibitory effect on cell proliferation. The anti-tumor activity of IFN- β 1a was significantly higher than that of IFN- α 2b in both cell lines, which is related to the higher induction of apoptosis in H295 cells and S phase accumulation in both cell lines. The higher sensitivity to IFN- β 1a may be explained by differences in the structure and affinity between IFN- α and IFN- β . There is only 35% sequence identity between both IFNs, and unlike IFN- α , IFN- β 1a is glycosylated and includes a zinc ion (25, 26). These differences could generate differential interactions with the same receptor. In fact, although both IFNs induce tyrosine phosphorylation of the receptor subunits, IFN- β 1a, but not IFN- α , induces the association of IFNAR-1 and IFNAR-2c chains, indicating that the specificity of signaling for distinct type I IFN subtypes could be established by differential conformation of the receptor complex (26, 27). Finally, IFN- β has a higher affinity for the relative receptor than IFN- α (28).

H295 cells were significantly more sensitive to IFN- β 1a treatment compared with SW13. This could be a consequence of the IFN-mediated pro-apoptotic activity only in H295 cells, as shown by the increase in DNA fragmentation and in the percentage of cells in subG₀ phase after the treatment with IFN. We evaluated the role of the activation of caspases in the induction of DNA fragmentation after IFN- β 1a treatment in H295 cells. In the classical model, caspases are divided into initiator caspases (such as caspase-8, -9) and executioner caspases (caspase-3, -6, -7), according to their function and their sequence of activation. There are at least two major apoptotic pathways. The first involves the death receptor or extrinsic pathway, which is initiated by TNF-receptor family members that recruit adaptor and signaling molecules to

assemble the death-inducing signaling complex. This complex leads to activation of caspases 8 and/or 10. An alternative mitochondrial pathway involves activation of caspase-9 upon recruitment to the mitochondria by cytochrome c and apoptosis protease activation factor-1. More downstream, the initiator caspases lead to the activation of executioner caspases -3, -6, and -7, which in turn cleave specific proteins resulting in the DNA fragmentation (29, 30). In H295 cells the stimulation of DNA fragmentation by IFN- β 1a is completely blocked by a specific inhibitor of caspase-3, while is partially counteracted by the use of specific inhibitors for caspase-8 and -9. On these bases, IFN- β 1a seems to induce apoptosis through both the extrinsic and mitochondrial pathways in ACC.

At present, two recombinant IFNs- β (IFN- β 1a and IFN- β 1b) are available as registered drugs. IFN- β -1a is produced in mammalian cells, with an amino acid sequence and glycosylation identical to that of natural IFN- β . In contrast, IFN- β 1b is produced in *Escherichia coli* bacteria, and as bacteria lack the ability to glycosylate proteins, it is not glycosylated. Furthermore, IFN- β 1b has one amino acid sequence different from the natural human IFN- β ; it has a cysteine-to-serine substitution at position 17 and a deletion of the *N*-terminal methionine residue. We observed in H295 cells that the effects of IFN- β 1a on cell proliferation inhibition and stimulation of DNA fragmentation were much more potent compared with IFN- β 1b. This is in agreement with the observation that the absence of glycosylation in IFN- β -1b can reduce the biological activity (31).

Finally, we evaluated the effects of IFN- β 1a on IGF-II mRNA expression, the most important autocrine/paracrine growth factor for ACC. H295, but not SW13 cells express IGF-II. IGF-II mRNA was inhibited in a dose dependent manner by IFN- β 1a after 3 days of incubation. This effect was not observed after incubation with IFN- α . It is well known that in ACC the up-regulation of the IGF-II system represents a main pathway involved in the pathogenesis, through the stimulation of the proliferation and inhibition of apoptosis (32). This could partially explain why only in H295 cells IFN- β 1a is able to induce apoptosis.

In conclusion, this is the first study showing that type-I IFNs, particularly IFN- β 1a, have a potent inhibitory effect on the proliferation of the H295 and SW-13 human ACC cells. This effect is mediated via the induction of apoptosis and/or a cell cycle arrest in the S-phase. Our findings support the clinical attractiveness to use IFN- β in the treatment of ACC, considering that IFN- β inhibited cell proliferation and stimulated apoptosis already at very low concentrations (1-10 IU/ml). In addition, IFN- β 1a is able to inhibit at

transcriptional level the expression of IGF-II. Further studies are necessary to evaluate *in vivo* this promising drug, not only as a single compound but also in combination with the currently used cytostatic mitotane.

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CHAPTER V:

EGF ACTIVATES AN INDUCIBLE SURVIVAL RESPONSE VIA THE RAS-> ERK-1/2 PATHWAY TO COUNTERACT INTERFERON- α -MEDIATED APOPTOSIS IN EPIDERMOID CANCER CELLS



Caraglia M, Tagliaferri P, Marra M, Giuberti G, Budillon A, Di Gennaro E, Pepe S, **Vitale G**, Improta S, Tassone P, Venuta S, Bianco AR, Abbruzzese A. *Cell Death and Differentiation* 2003;10:218-229.

Abstract

The mechanisms of tumor cell resistance to interferon- α (IFN- α) are at present mostly unsolved. We have previously demonstrated that IFN- α induces apoptosis on epidermoid cancer cells and EGF antagonizes this effect. We have also found that IFN- α -induced apoptosis depends upon activation of the NH₂-terminal Jun kinase-1 (Jnk-1) and p38 mitogen-activated protein kinase, and that these effects are also antagonized by EGF. At the same time, IFN- α increases the expression and function of the epidermal growth factor receptor (EGF-R). Here we report that the apoptosis induced by IFN- α occurs together with activation of caspases 3, 6 and 8 and that EGF also antagonizes this effect. On the basis of these results, we have hypothesized that the increased EGF-R expression and function could represent an inducible survival response that might protect tumor cells from apoptosis caused by IFN- α via extracellular signal regulated kinase 1 and 2 (Erk-1/2) cascades. We have found an increased activity of Ras and Raf-1 in IFN- α -treated cells. Moreover, IFN- α induces a 50% increase of the phosphorylated isoforms and enzymatic activity of Erk-1/2. We have also demonstrated that the inhibition of Ras activity induced by the transfection of the dominant negative Ras plasmid RASN17 and the inhibition of Mek-1 with PD098059 strongly potentiates the apoptosis induced by IFN- α . Moreover, the selective inhibition of this pathway abrogates the counteracting effect of EGF on the IFN- α -induced apoptosis. All these findings suggest that epidermoid tumor cells counteract the IFN- α -induced apoptosis through a survival pathway that involves the hyperactivation of the EGF-dependent Ras \rightarrow Erk signalling. The selective targeting of this pathway appears to be a promising approach in order to enhance the antitumor activity of IFN- α .

Introduction

Interferon- α (IFN- α) is a biological agent widely used in the therapy of several neoplasms such as myeloma, renal cell carcinoma, epidermoid cervical and head and neck tumors and melanoma (1–5). IFN- α , the first cytokine to be produced by recombinant DNA technology, has emerged as an important regulator of cancer cell growth and differentiation, affecting cellular communication and signal transduction pathways, and is also produced during the immunological control of tumor growth (5). However, inconsistent data have been obtained regarding the clinical effectiveness of IFN- α in the therapy of solid tumors. In fact, the benefit of IFN- α treatment is limited to some neoplasms while others are completely or partially resistant. The mechanisms of tumor resistance to IFN- α have been studied *in vitro*. The alteration of JAK-STAT components of the IFN- α -induced signalling can be indeed a mechanism of resistance to IFN. In fact, it has been shown that melanoma cell lines refractory to the antiproliferative effects of IFNs are deficient in STATs and that the expression of STATs can be restored by *in vitro* gene therapy (6). More germane to clinical practice is the possibility that IFN treatment could be improved by the concomitant administration of agents known to enhance JAK-STAT responses; the use of retinoids in combination with IFN- α in cancer therapy is a salient example (7,8).

The way in which tumor cell growth is suppressed by IFN- α is not well known. A possibility is that cancer cells undergo apoptosis after exposure to the cytokine. In fact, it is reported that IFN- α induces apoptosis in human squamous cancer (9,10), glioma (11) and virus-infected cells (12). Therefore, it is likely that this cytokine acts, at least in part, through the triggering of programmed cell death.

On the other hand, Epidermal Growth Factor (EGF) is able to protect eukaryotic cells from the onset of apoptosis (13–16). EGF acts through the binding to its specific receptor (EGF-R), a transmembrane protein with a cytoplasmic tyrosine kinase domain (17,18). The phosphorylation of the intracytoplasmic tail allows the interaction of EGF-R with cytoplasmic factors that can induce Ras activation and the subsequent stimulation of the mitogen-activated protein kinase (MAPK) cascade composed of three intracellular protein kinases (MKKK, MKK and MAPK), which are activated successively by phosphorylation events (17,18). Raf-1, stimulated after steric interaction with Ras, phosphorylates and activates an MKK whose main component is Mek-1. Mek-1 phosphorylates the MAPKs extracellular signal regulated kinase 1 and 2 (Erk-1/2) that translocate to the nucleus and phosphorylate gene transactivators, such as the serum response factor-1, that are involved in the regulation of cell proliferation (17,18). Moreover, it has been recently

reported that Erks mediate a strong antiapoptotic effect (19,20). For instance, Erk plays an important role in protecting cardiac myocytes from apoptotic death following oxidative stress (21). Protection of PC12 cell death by N-acetylcysteine requires Erk activation (20) that is, moreover, involved in the phorbol myristate acetate-mediated inhibition of drug-induced apoptosis in tumor cells (22).

We have reported that IFN- α increases the functional expression of the EGF-R at the surface of human epidermoid carcinoma cells (23,24).

Moreover, we have shown that IFN- α induces apoptosis in these cells through the triggering of a stress response that leads to the activation of NH₂-terminal Jun kinase-1 (Jnk-1) (10). EGF also antagonizes these effects. On the basis of these findings, we have hypothesized that increased EGF-R expression and function could be part of an inducible survival pathway, which is activated in the tumor cells by exposure to IFN- α . In this paper, we have investigated the molecular basis of the counteracting effects of IFN- α and EGF on the apoptosis of human epidermoid cancer cells. Specifically, we have evaluated the involvement of the caspase cascade and of EGF-dependent signalling in the apoptotic and antiapoptotic events recorded in our experimental model. We have then studied the effects of IFN- α and EGF on the activity of Ras, Raf-1 and Erk-1/2. We have finally evaluated if the selective inhibition of Ras and Mek-1 could enhance apoptosis induced by IFN- α in order to provide direct evidence on the antiapoptotic activity of this pathway in our experimental model and to identify novel molecular targets to enhance the antitumor effects of this cytokine.

Materials and Methods

Materials

DMEM, BSA and FBS were purchased from Flow Laboratories (Milan, Italy). Tissue culture plasticware was from Becton Dickinson (Lincoln Park, NJ, USA). IFN- α was a gift of Schering (Schering-Plough, NJ, USA). Receptor grade EGF and protein Sepharose were purchased from Sigma (St. Louis, MO, USA). Rabbit antisera raised against Raf-1 C-12, β -actin, Erk-1 K-23 and Erk-2 MAb C-14 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-pan-Ras MAb clone 10 was purchased from Calbiochem.

Cell culture

The human oropharyngeal epidermoid carcinoma KB cell line, obtained from the American Type Tissue Culture Collection, Rockville, MD, USA, was grown in DMEM supplemented with heat-inactivated 10% FBS, 20mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1% L-Glutamine and 1% sodium pyruvate. The cells were grown in a humidified atmosphere of 95% air/5% CO₂ at 37°C.

Western blot analysis

KB cells were grown for 48 h with or without IFN- α and were thereafter exposed for the indicated times to 10 nM EGF at 37°C. For cell extract preparation, the cells were washed twice with ice-cold PBS/BSA, scraped, and centrifuged for 30 min at 4°C in 1 ml of lysis

buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 NaCl, 1mM EDTA, pH 7.5, 10mM Na_2HPO_4 , pH 7.4, 10mM PMSF, 25mM benzamidin, 1mM leupeptin, 0.025 U/ml aprotinin). Equal amounts of cell proteins were separated by SDS-PAGE. The proteins on the gels were electrotransferred to nitrocellulose and reacted with the different MABs.

Affinity precipitation of Ras

KB cells were treated with IFN- α and EGF as described above. The cells were lysed in Mg^{2+} buffer containing 20mM HEPES, pH 7.5, 150mM NaCl, 1% Igepal CA-630, 10mM MgCl_2 , 1mM EDTA and 2% glycerol. Then, 10 μl Ras binding domain (RBD) conjugated to agarose was added to 1mg of cell lysate and the mixture was incubated at 4°C for 1 h. The agarose beads were collected by microcentrifugation at 14,000 x g for 5 s and washed three times with Mg^{2+} buffer. The agarose beads were boiled for 5 min in 2 x Laemmli sample buffer and collected by a microcentrifuge pulse. The supernatants were run on 12% SDS-PAGE, then the proteins were electrotransferred on a nitrocellulose film. The nitrocellulose was incubated overnight with 1 $\mu\text{g}/\text{ml}$ of anti-Ras Mab, clone RAS10 and with a secondary Mab, a goat α -mouse HRP conjugated IgG, for 1.5 h. The film was washed with PBS/0.05% Tween 20 and detected by ECL, chemiluminescence's technique (Amersham).

Internucleosomal DNA fragmentation (ladder)

For all apoptosis evaluation experiments (gel ladder and FACS analysis), both attached and suspended cells were collected prior to the processing. DNA fragmentation was measured after extraction of low molecular weight DNA. Briefly, 10×10^6 cells were resuspended in 900 μl x Tris-EDTA buffer and lysed with 25 μl 20% SDS. DNA was precipitated in ethanol for 6 h in the presence of 5M NaCl. The high molecular weight fraction was sedimented by high-speed centrifugation, and the fragmented DNA was extracted from the aqueous phase with phenol and chloroform and then precipitated with ethanol. After resuspension in water, DNA was electrophoresed using 1.5% agarose gel and visualized by ultraviolet light following ethidium bromide staining.

Evaluation of apoptosis by DNA-flow cytometry

Cells were centrifuged and directly stained in a propidium iodide (PI) solution (50 μg PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) overnight at 4°C in the dark. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) interfaced with a Hewlett-Packard computer (mod. 310) for data analysis. To evaluate cell apoptosis, PI fluorescence was collected as FL2 (log scale) by the CellFIT software (Becton Dickinson). For the evaluation of apoptosis after transfection with RASN17 and/or β -galactosidase plasmids, cells were fixed in methanol and then incubated with an anti- β -galactosidase MAB for 1 h at 4°C and labelled with an FITC anti-mouse rabbit antibody for 1 h. Thereafter, the cells were stained with PI as described above. In the latter case, apoptosis and cell cycle analysis were performed on the whole cell population and on β -galactosidase expressing cells after appropriate gating. For the evaluation of intracellular DNA content, at least 10,000 events for each point were analyzed in at least three different experiments giving a s.d. less than 5%.

MAPK assay

KB cells were cultured and treated as described above. Then the cells were washed twice with ice-cold PBS, scraped and lysed for 1 h at 4°C in the following buffer: 10mM TRIS, 150mM NaCl, 2mM EGTA, 2mM DTT, 1mM sodium orthovanadate, 1mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1% glycerol. Equalized amounts of proteins from lysates of KB cells (50 μl) were incubated for 90 min at 4°C with 50 μl of protein A-Sepharose conjugated to 25 μg anti-Erk-1 or anti-Erk-2 MABs. The suspension was centrifuged at 14,000 x g, the supernatant discarded, and the immunoprecipitated washed twice with 250 μl of the following buffer: 20mM Tris-acetate, pH 7.0 (20°C), 0.27M sucrose, 1mM EDTA, 1mM EGTA, 1mM sodium orthovanadate, 10mM sodium glycerophosphate, 50mM sodium fluoride, 5mM sodium pyrophosphate, 1% Triton X-100, 0.1% 2-mercaptoethanol, 1mM benzamidine, 0.2mM phenylmethylsulfonylfluoride and 0.5M NaCl. The immunoprecipitates were washed once with 250 μl of the following buffer: 50mM Tris-HCl, pH 7.5 (20°C), 0.03% Brij-35, 0.1mM EGTA and 0.1% 2-mercaptoethanol. Then the immunoprecipitates

were incubated for 30 min at 30°C with 5 ml of a solution containing 1.2mM Mg⁺⁺ ATP with 200 µCi/ml of [γ^{32} P]ATP and 15 µl of a solution containing a peptide that is specifically phosphorylated by MAPK (Amersham, Biotrak, Milan, Italy) (25). The reaction was stopped with the addition of 2.94% (w/v) orthophosphoric acid and red carmosin solution and then microfuged for 15 s. The reaction mixtures were spotted onto phosphocellulose filters (Whatman P81) and washed three times in 1% acetic acid. Filters were air-dried and then counted by liquid scintillation using Omnifluor/toluene (DuPont-New England Nuclear, Boston, MA, USA).

Raf-1 kinase assay

Assay for Raf-1 kinase activity was performed by immunocomplex kinase assay as already described for MAPK using anti-Raf-1 antiserum. H1 histone (10 µg) (Upstate Biotechnology Inc.) was used as substrate in 30 µl of buffer A containing 10 µCi of [γ^{32} P]ATP, the reaction was allowed to proceed for 30 min at 30°C, and the proteins were separated by SDS-12.5% PAGE.

Electroporation

Cells were detached from confluent 100 mm- dishes. Cells (100x10⁶) were incubated in appropriate electroporation vials with 800 µl of electroporation buffer (20mM HEPES, 137mM NaCl, 5mM KCl, 0.7mM Na₂HPO₄ and 6mM glucose) and 15 µg of the RASN17 DNA and/or 5 µg/ml β -galactosidase DNA in 20mM HEPES. Then cells were electroporated at 250 V and at 975 µF for 6 s. The cells were incubated at 37°C with or without 1000 IU/ml IFN- α for 24 h. After the incubation, the cells were processed for FACS analysis as described above.

TUNEL technique

For TUNEL assay, after washing in PBS supplemented in 0.1% BSA, cells were treated with an *in situ* detection kit, according to the manufacturers instructions (Boehringer Mannheim Biochemicals). Nuclei with fragmented DNA were visualized by a fluorescence microscope.

Determination of caspase activity

Cells were seeded and treated with 1000 IU/ml IFN- α a for different times and/or 10 nM EGF for 12 h. At the time of caspase determination, cells were lysed. For each reaction, 50 µl of cell lysate was added to 1ml reaction mixture composed of reaction buffer, DTT 10mM and the specific substrate peptide (DEVD for caspase-3, VEID for caspase-6, IETD for caspase-8 and LEHD for caspase-9) conjugated to 7-amino-4-trifluoromethylcoumarin (AFC). The mixtures were incubated for 1 h at 37°C. The levels of free AFC were measured using a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength range of 480–520 nm (peak at 505 nm).

Results

Effects of IFN- α and EGF on the apoptosis of human epidermoid cancer cells.

We have found that IFN- α (48 h 1000 IU/ml) induced apoptotic DNA fragmentation in the KB cell line and that the exposure of IFN- α -treated cells to 10nM EGF for 12 h antagonized this effect (fig.1a). Before performing apoptosis detection assays, both attached and suspended cells were collected. In these experimental conditions, 30% of cells exposed to IFN- α for 48 h were apoptotic while only about 8% of cells treated for 48 h with IFN- α and exposed for 12 h to 10 nM EGF underwent to apoptosis (fig.1b). Interestingly, also the exposure to 10nM EGF alone for 12 h is able to induce apoptosis in

15% of the cell population (fig.1a and b). Such an effect is not surprising since apoptosis and cell proliferation are couplet processes, and EGF can induce apoptosis in squamous cancer cells. In order to identify the molecular effectors of the apoptosis induced by IFN- α , we have evaluated the expression of p53 and c-myc and the activity of caspases in KB cells. We have found that apoptosis induced by either IFN- α or EGF was paralleled by a significant increase of p53 and c-myc expression in KB cells, as evaluated with Western blot assay. The antagonism of EGF on apoptosis occurred together with the restoration of p53 and c-myc expression to basal levels (fig.1C).

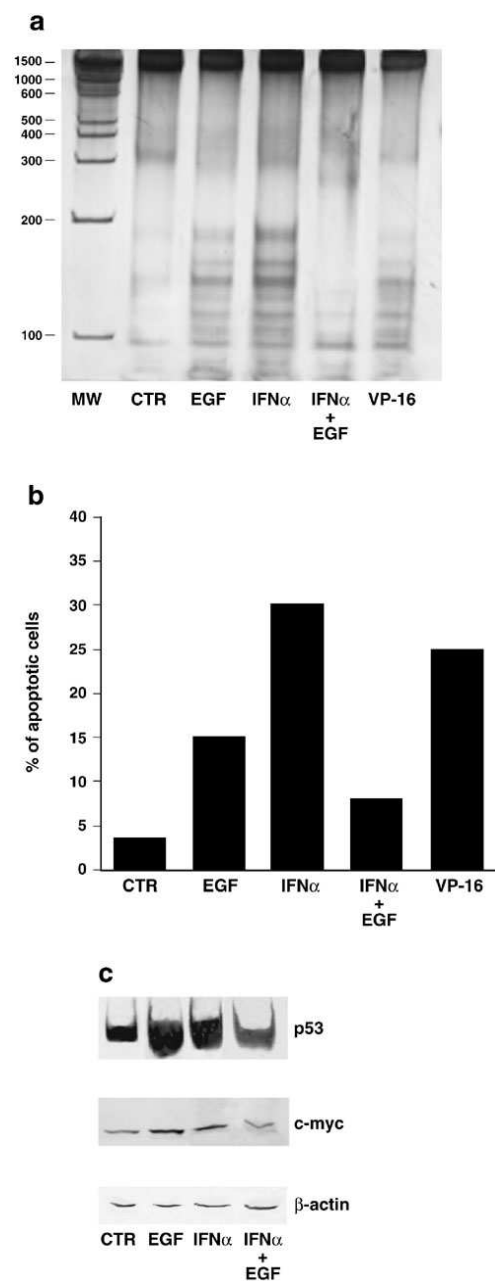


Figure 1: EGF antagonizes apoptosis induced by IFN- α in human epidermoid cancer cells. KB cells were seeded and treated with IFN- α and/or EGF as described in the Materials and methods section.

(a) The internucleosomic DNA fragmentation was assessed as described in the Materials and methods section.

(b) The percent of apoptotic cells, evaluated by FACS analysis, is shown as bars. **CTR:** untreated cells; **IFN- α :** 48 h 1000 IU/ml; **EGF:** 12 h 10 nM; **IFN- α + EGF:** 48 h 1000 IU/ml IFN- α + 12 h 10 nM EGF. The experiment was performed three times and S.E.s were always less than 5%.

(c) Evaluation of the expression of p53, c-myc and β -actin. Proteins were extracted and run on SDS-PAGE as described in 'Materials and methods section'. The experiment was performed at least three times and the results were always similar. **CTR:** untreated cells; **IFN- α :** 48 h 1000 IU/ml; **EGF:** 12 h 10 nM; **IFN- α +EGF:** 48 h 1000 IU/ml IFN- α +12 h 10 nM EGF; **VP-16:** 18 h 50 μ M etoposide.

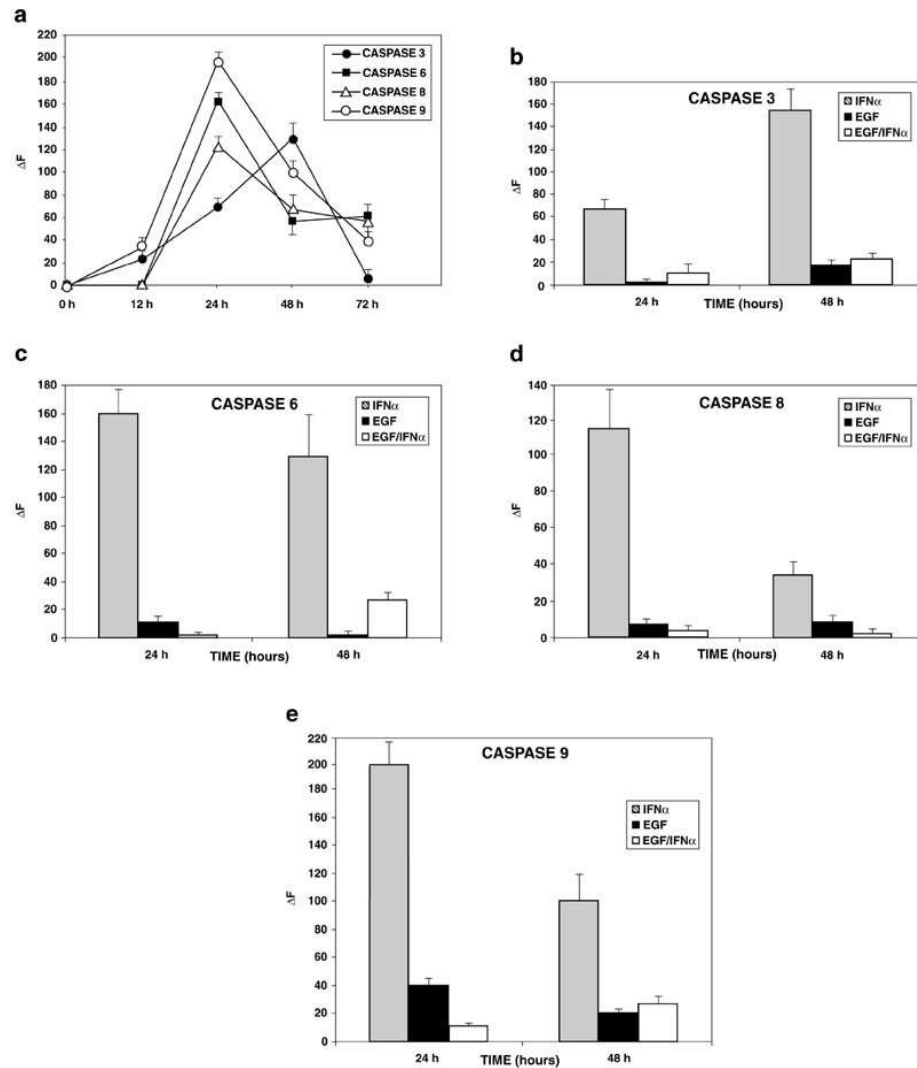


Figure 2: Modulation of caspases 3, 6, 8 and 9 by IFN- α and EGF. **(b)** KB cells were seeded and treated with 1000 IU/ml IFN- α for the indicated times. The specific caspase activity was determined with a fluorimetric assay as described in the 'Materials and methods section'. The experiment was performed three times and S.D.s were always less than 5%. Bars, s.d. **(b-d)** KB cells were seeded and treated with 1000 IU/ml IFN- α for the indicated times. EGF (10 nM) was added to the cells for the last 12 h of incubation alone or in combination with IFN- α . The specific caspase activity was determined with a fluorimetric assay as described in the 'Materials and Methods section'. Caspase 3 **(b)**; caspase 6 **(c)**; caspase 8 **(d)**; caspase 9 **(e)**. 1000 IU/ml IFN- α (■); 10nM EGF (■); IFN- α +12 h 10 nM EGF (□). The experiment was performed three times and S.D.s were always less than 5%. Bars, S.D.

We have thereafter studied the role of caspases in the modulation of apoptosis by IFN- α and EGF in our experimental model. KB cells were treated with IFN- α for 12, 24, 48 and 72 h and then the activity of caspases was evaluated with a fluorimetric protease assay. Caspases 6, 8 and 9 showed an activation peak after 24 h exposure to IFN- α (fig.2a). The activation peak of caspase 3 was instead found after 48 h exposure to the cytokine. Moreover, the exposure of KB cells to 10nM EGF for 12 h was always able to antagonize the caspase

activation induced by IFN- α both at 24 h and 48 h (fig.2b-d). In fact, EGF completely abrogated caspases 6, 8 and 9 and caspase 3 activities. EGF alone also induced a slight increase of caspase stimulation, but the extent of activation was far lesser than that recorded in IFN- α -treated cells. Therefore, in our experimental model the IFN- α -induced apoptosis is correlated with the activation of the caspase cascade, and EGF was able to antagonize both apoptotic death and caspase activation. Moreover, the apoptosis recorded in KB cells exposed to EGF alone did not appear to be induced by a caspase pathway similar to that recorded in IFN- α -treated cells.

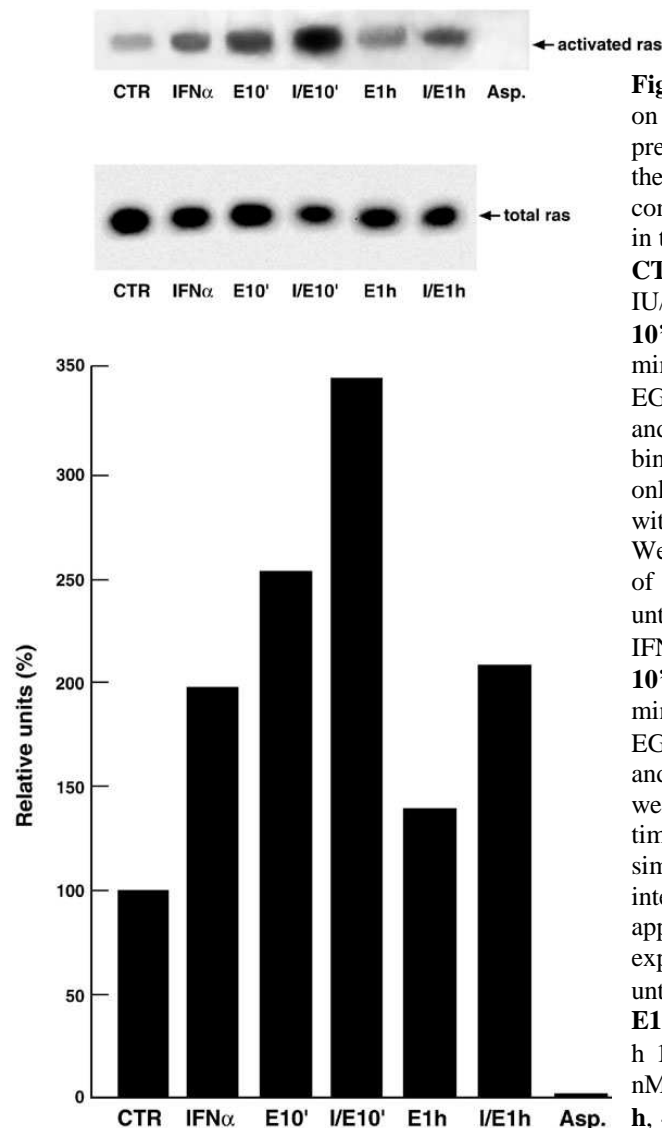


Figure 3: Effects of EGF and IFN- α on Ras activity. (Upper gel) Affinity precipitation of Ras performed with the minimal binding domain of Raf-1 conjugated with agarose as described in the 'Materials and methods section'. **CTR**, untreated; **IFN- α** , 48 h 1000 IU/ml; **E10'**, 10 min 10 nM EGF; **I/E 10'**, 48 h 1000 IU/ml IFN- α and 10 min 10 nM EGF; **E1 h**, 1 h 10 nM EGF; **I/E 1 h**, 48h 1000 IU/ml IFN- α and 1 h 10 nM EGF; **Asp**, minimal binding domain of Raf-1 incubated only with Mab raised against Ras without cell extracts. (Lower gel) Western blot assay for the expression of the total Ras protein. **CTR**, untreated; **IFN- α** , 48 h 1000 IU/ml IFN- α ; **E10'**, 10 min 10 nM EGF; **I/E 10'**, 48 h 1000 IU/ml IFN- α and 10 min 10 nM EGF; **E1 h**, 1 h 10 nM EGF; **I/E 1 h**, 48 h 1000 IU/ml IFN- α and 1 h 10 nM EGF. The experiments were performed at least three different times and the results were always similar. (Bars) Quantitization of the intensity of the different bands with an appropriate apparatus (Bio-Rad) and expression as arbitrary units. **CTR**, untreated; **IFN- α** , 48 h 1000 IU/ml; **E10'**, 10 min 10 nM EGF; **I/E 10'**, 48 h 1,000 IU/ml IFN- α and 10 min 10 nM EGF; **E1 h**, 1h 10nM EGF; **I/E 1 h**, 48 h 1000 IU/ml IFN- α and 1 h 10 nM EGF; **Asp**, minimal binding domain of Raf-1 incubated only with Mab raised against Ras without cell extracts.

Effects of EGF and IFN- α on Ras activity

We have previously shown that IFN- α induces growth inhibition paralleled by increased EGF-R expression and sensitivity to the growth-promoting effects of EGF in epidermoid cancer cells (23,24). Therefore, we have investigated if the activity of the Ras-dependent MAPK pathway was increased in IFN- α treated cells. This and all the following experiments were performed without serum starvation in order to set up more physiological experimental conditions. Moreover, EGF was added for short-term exposures in order to evaluate the responsiveness of each signalling component to its physiological ligand in untreated and IFN- α -treated KB cells. We have firstly studied the activity of Ras through the affinity precipitation of Ras with the minimal binding domain of Raf-1. We have found that the levels of activated Ras (linked to the minimal binding domain of Raf-1) were increased in IFN- α -treated cells. In fact, the activated Ras levels were about two-fold higher in IFN- α -treated cells than in control cells, and the stimulation of Ras activity by exposure to EGF for 10 min was further enhanced in IFN- α -treated cells reaching levels 3.5-fold higher than untreated controls (Figure 3). At longer times of exposure to EGF, Ras activity decreased either in EGF- or EGF/IFN- α treated cells (Figure 3). Therefore, in IFN- α -treated KB cells, the increased activity of EGF-R signalling was indeed coupled to enhanced Ras activity and was further stimulated by the addition of exogenous EGF.

Effects of IFN- α and EGF on Raf-1 expression and activity

We have evaluated the effect of IFN- α and EGF on Raf-1 activity by a kinase assay using histone H1 as substrate of Raf-1, and we have found that Raf-1 activity is increased either by EGF or IFN- α and is potentiated after EGF addition for 10 min to KB cells exposed to IFN- α (fig.4). Moreover, we have determined Raf-1 levels by Western blotting, and found that they were almost unmodified by the different treatments (fig.4). These experiments demonstrate that an increase of basal and EGF-induced enzymatic activity of Raf-1 can be recorded downstream of the EGF->Ras signalling in IFN- α -treated cells.

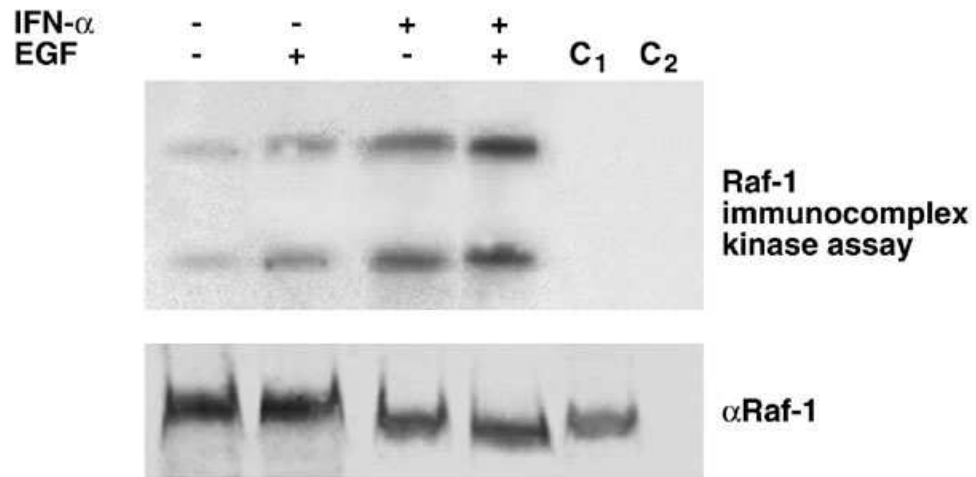


Figure 4: Effects of IFN- α and EGF on Raf-1 activity and expression. KB cells have been cultured for 48 h in the absence or presence of 1000 IU/ml IFN- α with or without the addition of 10 nM EGF for the last 5 min as described in the figure by + and -. Raf-1 was immunoprecipitated and analyzed for kinase activity by immunocomplex kinase assay using histone H1 as substrate. Lane C1 indicated a control where the immunocomplex kinase assay was performed on untreated EGF-stimulated cells in the absence of the substrate. Lane C2 indicated a control assay performed using nonimmune serum to immunoprecipitate untreated EGF-stimulated cells. At the same time, the cells were also processed for the determination of Raf-1 expression as described in the ‘Materials and Methods section’.

Effects of IFN- α and EGF on Erk-1/2 activity

On the basis of the finding that IFN- α induced a hyperactivation of Mek-1, we have evaluated the effects of this cytokine on the activity of Erk-1/2, the downstream enzymatic targets of Raf-1. We found that the exposure of KB cells to IFN- α for short times (5 min-12 h) had no effects on MAPK activity, while they were maximally activated after 48 h of treatment with the cytokine (fig.5a, lower panel). The latter effect paralleled EGF-R upregulation, which reached a peak after 48 h of exposure to IFN- α (fig.5a, upper panel and see also ref. 24). Therefore, the timing of MAPK²⁵ activation by IFN- α overlapped that of EGF-R upregulation, suggesting that the stimulation of MAPK activity could be a consequence of the increased surface expression of EGF-R with subsequent amplification of downstream signalling. We have subsequently evaluated the kinetics of Erk-1/2 activation by EGF in untreated and IFN- α -treated KB cells. We again found an almost two-fold increase of the two enzyme activities in 48 h 1000 IU/ml IFN- α -treated cells. In both untreated and 48 h 1000 IU/ml IFN- α -treated KB cells, the maximal activation of Erk-1/2 occurred after 10 min of exposure to 10nM EGF, as evaluated with an immunoconjugated kinase assay (fig.5b and c). However, the exposure to IFN- α caused an almost two-fold increase of the maximal

activation of Erk-1/2 induced by EGF (fig.5b and c). Therefore, 48 h exposure to IFN- α increased the activity of Erk-1/2 without affecting the sensitivity to further stimulation by exogenous EGF.

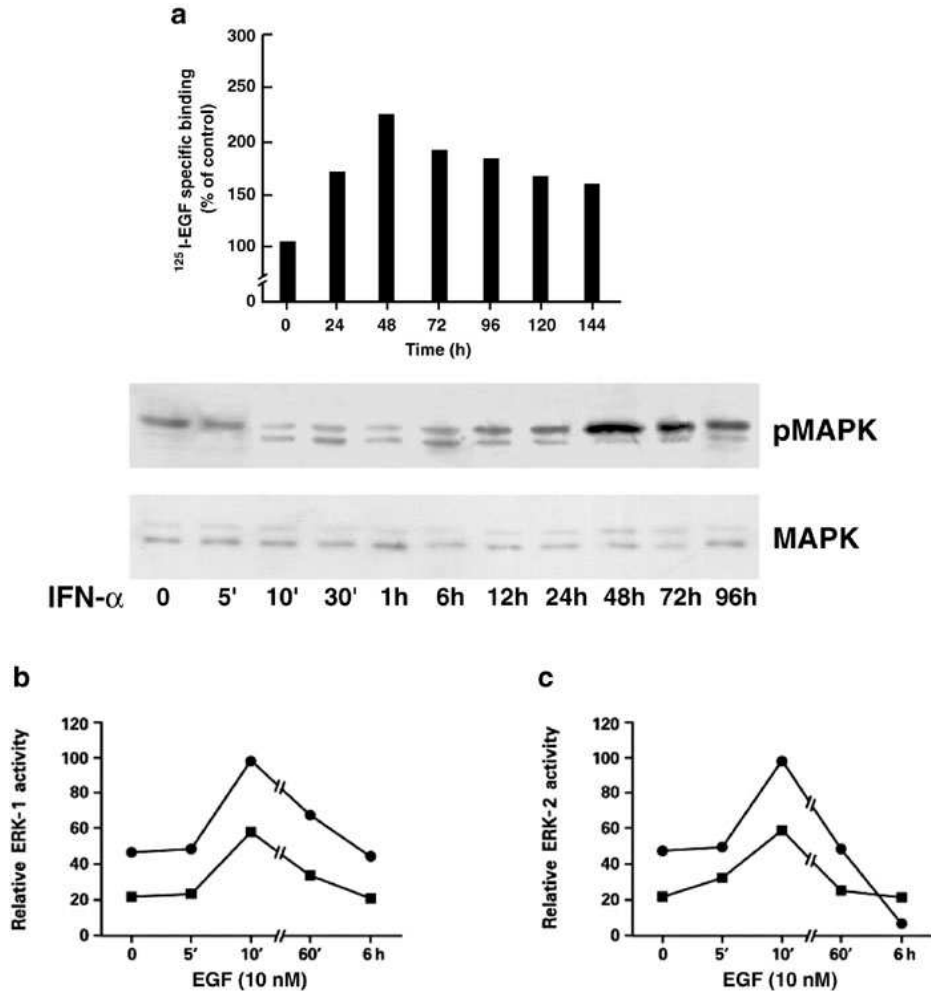


Figure 5: IFN- α increases Erk-1 and Erk-2 activity in human epidermoid cancer cells. (A) upper panel) ^{125}I -EGF binding on human epidermoid KB cells. KB cells were treated for different times with 1000 IU/ml IFN- α . At the indicated times, cells were processed for the determination of the ^{125}I -EGF specific binding, as described in the 'Materials and methods section'. The values were expressed as % of control. The experiments were performed three times and SDs were always less than 5%. (a, lower panel) KB cells were cultured for the indicated times in the absence or presence of 1000 IU/ml IFN- α . Then the cells were processed for the determination of the expression and phosphorylation of Erk-1 and -2 evaluated after blotting with an anti-MAPK and an anti-pMAPK specific Mab, respectively, as described in the 'Materials and methods section'. The experiments were performed at least three different times and the results were always similar. (b and c) KB cells were cultured for 48 h in the absence or presence of 1000 IU/ml IFN- α with or without the addition of 10 nM EGF for different times. Then an immunconjugated kinase assay of (b) Erk-1 and (c) Erk-2 was performed as described in 'Materials and Methods section'. Untreated KB cells (■); IFN α -treated KB cells (●). The experiment was performed three times and s.d.s were always less than 5%. Bars, s.d.

IFN- α enhanced the apoptosis induced by dominant negative Ras plasmid RASN17 in KB cells

We have shown that the EGF-dependent Ras->Erk pathway was hyperactivated in cells exposed to IFN- α . In order to demonstrate that such signalling could indeed retain an antiapoptotic activity in the cells exposed to IFN- α , we have selectively inhibited Ras activity by a dominant negative mutant. KB cells have been cotransfected with a plasmid encoding for a dominant negative Ras RASN17 and with the E. coli β -galactosidase reporter gene. The cells were analyzed by FACS after PI labelling as described above. In all cases, the cotransfection resulted in specific green fluorescence in a fraction of the cell population owing to the expression of β -galactosidase (see fig.6 β -Gal NEG versus β -Gal POS). The latter effect allowed us the gating of nontransfected (G2) or transfected cells (G1) in order to perform cell cycle analysis (red fluorescence determined by PI labelling). We have found that β -galactosidase expression alone did not cause apoptosis (2 and 6% of apoptosis either in nontransfected or β -galactosidase-transfected cells, respectively) (fig.6A, G2 and G1). In the cells cotransfected with RASN17 and β -galactosidase almost 29% of apoptotic cells were detected (fig.6B, G1), while only 2% of apoptosis was found in nontransfected cells (fig.6B, G2). The exposure of β -galactosidase-transfected cells to 1000 IU/ml IFN- α for 24 h induced apoptosis in 16% of the cell population (fig.6C, G1) against 14% of apoptotic cells in nontransfected population (fig.6C, G2). The treatment of β -galactosidase and RASN17 cotransfected cells with 1000 IU/ml IFN- α induced an almost 76% of apoptosis (fig.6D, G1), while programmed cell death was found in only 13% of the nontransfected cell population (fig.6D, G2). We have performed these experiments after 24 h of exposure to IFN- α because at this time maximal β -galactosidase expression occurred. Moreover, almost 80% of cells were apoptotic after 48 h from the transfection with RASN17 (data not shown). In this experiment about 30% of the cell population was transfected with β -galactosidase plasmid. It was therefore demonstrated that the abrogation of Ras activity by a transfected dominant negative mutant sensitized tumor cells to IFN- α -induced apoptosis, indicating an antiapoptotic function of Ras in this experimental model.

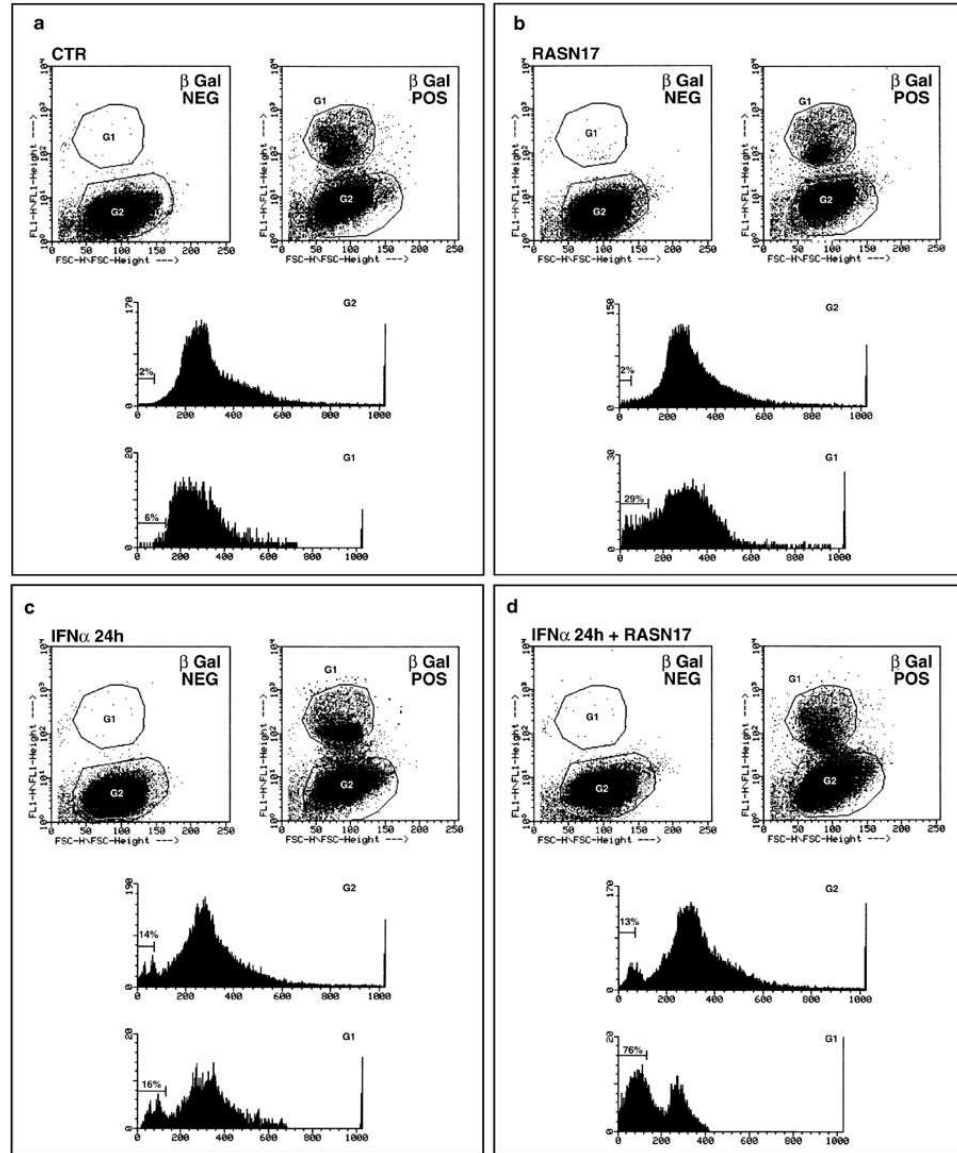


Figure 6 IFN- α enhances the apoptosis induced by the dominant negative Ras plasmid RasN17 in KB cells. KB cells were seeded, electroporated with dominant negative Ras plasmid RASN17 and/or β -galactosidase plasmid, and treated with IFN- α for 24 h as described above. Then the cells were fixed in methanol, incubated with anti- β -galactosidase MAb and anti-mouse FITC rabbit antiserum and analyzed after DNA labelling with propidium iodide. Cell cycle analysis was performed after appropriate gating on cell population expressing β -galactosidase as described in the ‘Materials and methods section’. (a) Parental untreated cells (CTR); (b) RASN17 transfected cells (RASN17); (c) 24 h 1000 IU/ml IFN- α -treated parental cells (IFN- α 24 h); (D) 24 h 1000 IU/ml IFN- α -treated RasN17 transfected cells (IFN- α 24 h+RASN17). β Gal NEG, cells exposed to an irrelevant mouse IgG and subsequently labelled with an anti-mouse FITC rabbit antiserum. β Gal POS, cells exposed to an anti- β -galactosidase mouse IgG and subsequently labelled with an anti-mouse FITC rabbit antiserum. G1, gating of cells expressing β -galactosidase; G2, gating of cells not expressing β -galactosidase. The lower histograms show the analysis of cell cycle performed on the G2 and G1 population, respectively. The apoptotic cells were shown as a percentage of total cell population indicated by a bar. The experiments were performed at least three different times and the results always gave less than 5% s.d. In each experimental point 10,000 events were analyzed.

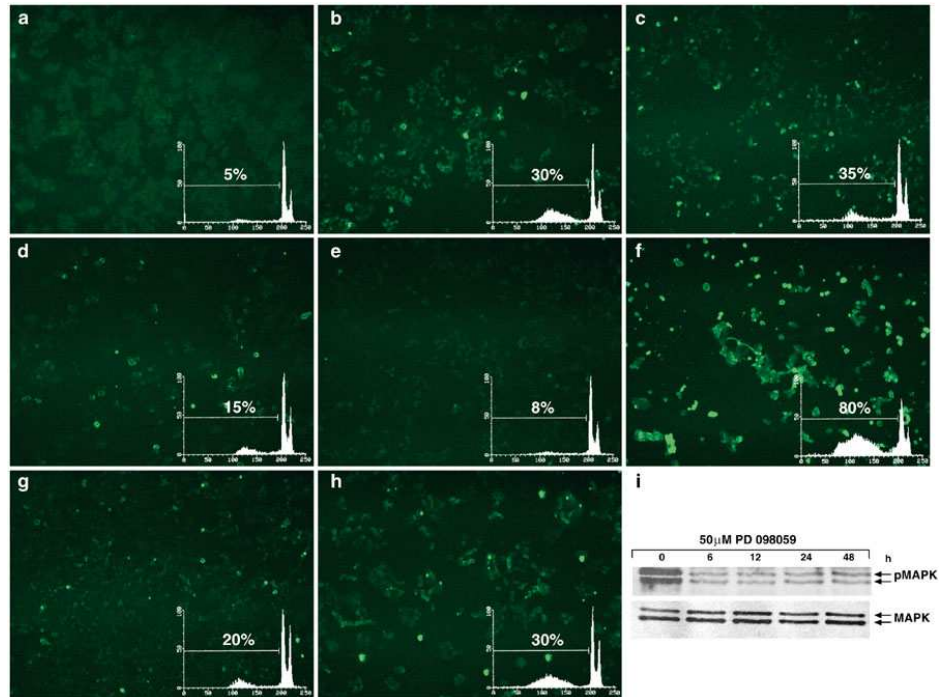


Figure 7 The antiapoptotic effects of EGF are mediated by the selective activation of Erk. KB cells were seeded and treated with IFN- α and/or 50 μ M PD098059 for 48 h and/or 10 nM EGF for 12 h. At the time of the experiment, cells were fixed and processed with TUNEL techniques as described in the ‘Materials and methods section’. The experiments were performed at least three different times and the results were always similar. (a) Control cells; (b) 48 h 1000 IU/ml IFN- α -treated cells; (c) 48 h 50 μ M PD098059-treated cells; (d) 12 h 10 nM EGF-treated cells; (e) 48 h 1000 IU/ml IFN- α and 12 h 10 nM EGF-treated cells; (f) 48 h 1000 IU/ml IFN- α and 48 h 50 μ M PD098059 treated cells; (g) 12 h 10 nM EGF- and 48 h 50 μ M PD098059-treated cells; (h) 48 h 1000 IU/ml IFN- α and 48 h 50 μ M PD098059-treated cells and 12 h 10 nM EGF. Insets: The cells were seeded and treated with the different substances as described above. At the time of the experiment KB cells were FACS analyzed after detachment and DNA labelling with propidium iodide as described in the ‘Materials and methods section’. The experiments were performed at least three different times and the results always gave less than 5% SD. In each experimental point, 10,000 events were analyzed. (i) The expression and phosphorylation of Erk-1 and -2 was evaluated after blotting with an anti-p-MAPK (upper gel) and an anti-MAPK-specific MAb (lower gel) in KB cells treated for different times with 50 μ M PD098059. The experiments were performed at least three different times and the results were always similar.

MEK-1 inhibitor PD098059 specifically abrogated recovery from IFN- α -induced apoptosis by EGF

Since Ras is implicated in the triggering of several different signal transduction pathways, we have verified that the integrity of the downstream Erk-targeted pathway was indeed required for the antiapoptotic effects of EGF (26). Therefore, we have evaluated if the specific inhibition of Mek-1 could also potentiate the growth inhibition and apoptosis induced by IFN- α and if the Ras->Erk-1/2 pathway plays a crucial role in the survival of epidermoid cancer cells exposed to IFN- α . Only 5% untreated KB cells were

apoptotic as assessed by FACS and in situ TUNEL analysis (fig.7A). Again 48 h 1000 IU/ml IFN- α induced 30% apoptosis and 12 h 10nM EGF antagonized this effect (only 8% apoptotic cells were recorded under these experimental conditions) (fig.7B and e and relative insets, respectively). In total, 50 μ M PD098059 and 10nM EGF alone induced 35 and 15% apoptosis, respectively (fig.7C and D, respectively). PD098059 was again able to enhance the effect induced by IFN- α causing programmed cell death in about 80% of cells, thus suggesting a supra-additive effect induced by the addition of MEK-1 inhibitor to IFN- α (fig.7F). Moreover, 50 μ M PD098059 abrogated the anti-apoptotic effects of EGF in IFN- α -treated cells since an almost 30% of apoptosis was recorded in cells exposed to IFN- α , EGF and PD098059 (fig.7H). On the other hand, PD098059 caused almost no effects on the apoptosis induced by EGF (fig.7G). Interestingly, PD098059 reduced MAPK phosphorylation almost completely already after 6 h in KB cells (fig.7I). All together, these data suggest that the antiapoptotic effects induced by EGF occurred through the activation of Erk, which is therefore the final target of an antiapoptotic kinase cascade.

Discussion

IFN- α is a cytokine that has shown a well-defined but still limited activity against human tumors (1–5). Resistance to IFN- α has been demonstrated to be in part based on receptor or post-receptor signalling defects in the tumor cells (6–8). However, the cancer cell escape from the antiproliferative activity of IFN- α and the mechanisms by which this cytokine causes direct antitumor effect remain still unsolved. Apoptosis induction appears to be a way by which IFN- α exerts its antiproliferative effects (9–12). It is well established that tumor cells overcome the natural propensity to undergo apoptosis with the overexpression and/or overactivation of growth factor-induced signal transduction pathways (13–15). EGF-R overexpression is a common event in epidermoid cancers and is considered an unfavorable prognostic factor (27). It has also been demonstrated that Erk-1/2 antagonize apoptosis in several experimental models (22,28–30). We have previously reported that IFN- α induces growth inhibition and increases the expression and function of EGF-R in human epidermoid cancer cells (23,24). Moreover, we have demonstrated that the growth inhibition induced by IFN- α is, at least in part, because of the apoptosis triggered by a stress response leading to the activation of JNK-1, and that EGF antagonizes growth inhibition, apoptosis and the biochemical

events induced by the cytokine (10). On the basis of these findings, we have hypothesized that the increased expression and function of the EGF-R could be part of a survival response that could protect tumor cells from the apoptosis triggered by IFN- α . In this paper, we have investigated the molecular bases of the counteracting effects of IFN- α and EGF on apoptosis. We have firstly studied the involvement of caspases in the execution of the apoptotic process. We have found that IFN- α induced an activation peak of caspases 6 and 8 after 24 h, while the maximal activation of caspase 3 was recorded 24 h later. The differential timing of caspase activation is in agreement with the sequence of activation of these proteases during the apoptotic process and suggests an involvement of caspases in the execution of apoptosis induced by IFN- α (31). Our findings are, moreover, in agreement with the recent observations by Thyrell et al. (32), who found induction of caspase activity by IFN- α in apoptotic tumor cells. In our experimental conditions, we have found that the addition of EGF to IFN- α -treated KB cells completely antagonized apoptosis induction and the triggering of the caspase cascade, suggesting that the EGF-R signalling suppresses apoptosis, thereby antagonizing the activation of its executioners. These results appear also in line with the recent findings demonstrating the involvement of growth-factor-dependent pathways in the protection from caspase activation induced by Bad overexpression. Moreover, it has been demonstrated that the EGF-R-dependent pathway controls keratinocyte survival and the expression of the proapoptotic bcl-xL expression through a Mek-dependent pathway (30). We have then investigated the involvement of EGF-mediated intracellular signalling in the protection of epidermoid cancer cells from IFN- α -induced apoptosis. We have found that the EGF- and Ras-dependent MAPK cascade is hyperactivated in IFN- α -treated cells and could be further stimulated by the addition of EGF. In fact, we have demonstrated an increased activity and responsiveness to EGF stimulation of Ras, Raf-1 and Erk-1/2 in KB cells exposed to IFN- α . These findings suggest that the EGF-R function is preserved in IFN- α -treated cells, taking also into account the activation threshold of the EGF-dependent signalling. Interestingly, maximal MAPK hyperactivation occurred after 48 h from the beginning of the treatment with IFN- α . It must be considered that the maximal upregulation of EGF-R on the cell surface occurs with the same timing, suggesting that overstimulation of the MAPK pathway could be produced by the amplification of the EGF signalling owing to the increased expression of the receptor (23,24). We have previously described that other antiproliferative agents, such as cytosine arabinoside, 5aza-2'deoxyctidine and 8-chlorocAMP (8ClcAMP), also increase EGF-R expression on KB cells (33–35). On this basis, we have

hypothesized that the upregulation of growth factor receptors is a common event in growth-inhibited tumor cells and could represent a protective response towards the antiproliferative stimuli (36). Also in the case of 8ClcAMP, the EGF-induced signalling MAPK is amplified likely as a consequence of the increased expression of EGF-R (35). However, MAPK activity is reduced in 8Cl-cAMP-treated KB cells, suggesting a selective inhibition of Erks or of a still unknown upstream activator induced by the drug (35). It has to be considered that chronic activation of Erk has been reported to induce apoptosis in eukaryotic cells (37–40). However, we can exclude that IFN- α -induced apoptosis occurs through the hyperactivation of Erks on the basis of both the timing of Erk activation and the effects of the combined treatment of KB cells with IFN- α and EGF. In fact, we have found that the exposure of IFN- α -treated KB cells to EGF did not simply induce a recovery of cell growth, but it also caused a proliferative effect much greater than that recorded in KB cells exposed to EGF alone (24).

The involvement of the Ras->MAPK pathway in the protection of KB cells from the apoptosis induced by IFN- α is further demonstrated by both Ras inactivation by RASN17 transfection and Mek-1 inhibition by exposure to PD098059. In fact, the transfection of RASN17 in KB cells caused apoptosis as demonstrated by the FACS analysis performed on transfected cells and also potentiated the apoptosis induced by IFN- α . These data suggest that the integrity of Ras function was necessary to produce an anti-apoptotic signal that mediates a survival response in cells exposed to IFN- α via Erk-1/2 activation. In fact, we have demonstrated that Ras-dependent survival signalling targets Erk-1/2 since the reduction of MAPK activity by PD098059 enhanced apoptosis caused by IFN- α . An additional important finding is that PD098059 specifically abrogated the recovery from apoptosis induced by EGF in IFN- α -treated cells. A second important antiapoptotic pathway involves signalling via Akt/PKB (41,42). In fact, it has been demonstrated that Akt can be activated concomitantly or independently from Ras->Erk-1/2 signalling by growth factors (43–45). Additionally, it has been reported that PC12 cells display a protective antiapoptotic pathway in response to hypoxic stimuli (46). The protection from apoptosis by Akt could be because of the regulation of mitochondrial physiology since Akt is involved in the regulation of bcl-related proteins such as Mcl-1 (43). However, the requirement of Akt for the protection from apoptotic events is highly variable depending upon the experimental model used (44,45,47). In our experimental conditions, we have demonstrated that PD098059 completely antagonized the antiapoptotic effects of EGF. Moreover, we have preliminarily found that the specific EGF-R-associated kinase inhibitor ZD1839 (IRESSA) synergizes with IFN- α in

inducing the growth inhibition and apoptosis of several human epidermoid cancer cell lines, which is coupled to complete inhibition of Erk activity (A Budillon et al., manuscript in preparation). Therefore, our results suggest that the activation of Ras->Raf-1->Mek1- >Erk-1/2 signalling has a prominent role in the antiapoptotic effects exerted by EGF in epidermoid cancer cells exposed to IFN- α , providing evidence of the potential benefits of the molecular interference with this pathway. However, the occurrence of other survival pathways will warrant further investigations and we cannot presently completely exclude a role of the Akt pathway in the modulation of apoptosis of KB cells.

Consistent with our results, additional findings have been recently reported on the interaction between the Ras->Erk pathway and the myc oncogene on apoptosis onset (48). In fact, it has been recently found that the activation of the Ras/Mek signalling suppresses myc-dependent apoptosis in cells with MAPK cascade amplification because of Ras activation (48). In our experimental model, we have also found increased expression of p53 and c-myc in cells exposed to IFN- α while the EGF anti-apoptotic effects were paralleled by downregulation of myc and p53 expression.

In conclusion, we have provided evidence that IFN- α makes the survival of human epidermoid cancer cells dependent on Ras/Erk signalling, which acts as a survival pathway. The specific disruption of the latter could be a useful approach to potentiate the antitumor activity of IFN- α against human epidermoid tumors as indicated by the results achieved by selective interference with Ras and Mek-1 function in cells exposed to IFN- α .

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CHAPTER VI:

GENERAL DISCUSSION



PARTS OF THIS CHAPTER ARE BASED ON:

Tagliaferri P, Caraglia M, Budillon A, Marra M, **Vitale G**, Viscomi C, Masciari S, Tassone P, Abbruzzese A, Venuta S. New pharmacokinetic and pharmacodynamic tools for interferon-alpha (IFN-alpha) treatment of human cancer. *Cancer Immunology Immunotherapy* 2005; 54: 1-10.

IFN- α is the first cytokine produced by recombinant DNA technology for clinical usage in the treatment of infectious as well as malignant diseases, through direct (cell growth inhibition) and indirect (immune stimulation) mechanisms.

In clinical practice, IFN- α has been used in the treatment of several cancers (1-15). In Table 1, we provide a general overview of IFN- α activity in human malignancies.

Table 1: IFN- α efficacy in human malignancies. RFS Relapse-free survival, OS overall survival, HR haematologic response, MS median survival

Tumor Type	Stage	Clinical Effects	Ref
Melanoma adjuvant	I Ib, III	Benefit in RFS and OS at 6.9 yrs	1
Melanoma adjuvant	I Ib, III	Benefit in RFS at 5.1yrs	2
Melanoma adjuvant	I Ib, III	Benefit in RFS and OS	3
Metastatic renal cell cancer	IV	Benefit in 1-yr survival and MS	4
Metastatic renal cell cancer	IV	Benefit in MS	5
Chronic myeloid leukemia		Benefit in HR rate and OS	6
Chronic myeloid leukemia		Benefit in OS	7
Multiple myeloma		Benefit in RFS and OS	8
Multiple myeloma		Benefit in RFS and OS	9

However, the use of IFN- α treatment in oncology is limited by 3 factors:

1. Several neoplasms are completely or partially resistant, even at high doses of IFN.
2. High-doses of IFN- α produce important toxicity, and at least 40% of patients require dose reduction and/or recycling delay due to severe toxicity, such as granulocytopenia, liver toxicity, neurological disorders and fatigue.
3. There are not molecular predictors of response potentially useful for deciding whether a patient should be treated with type I IFNs.

On the other hand, the efficacy of other type I IFNs has been poorly explored. Few authors reported that IFN- β has greater antitumor effects than IFN- α on melanoma, squamous carcinoma, glioma, breast and hepatocellular cancer (16-20). For these reasons the first aim of the present thesis (**chapter II, III, IV**) was to analyze the *in vitro* effects of IFN- α and IFN- β , the role of the relative receptors and the mechanisms of action in the following human cancer cell lines:

1. BON (neuroendocrine tumor of the pancreas)
2. BxPC-3, MiaPaCa-2 and Panc-1 (pancreatic adenocarcinoma)
3. H295 and SW13 (adrenocortical carcinoma).

In all these cell lines, the IFN receptor is expressed, as shown by quantitative RT PCR analysis and immunocytochemistry. Besides, the antiproliferative effect of IFN- β seems to be clearly more potent than IFN- α . The direct antitumor activity of IFN- β is modulated through the induction of apoptosis and an accumulation of the cells in the late part of S phase. The pro-apoptotic activity and the cell cycle arrest after IFN- β treatment are more potent and earlier than after IFN- α . In adrenocortical carcinomas (**chapter IV**) and in carcinoid cells (data not shown) IFN- β could stimulate the apoptosis through the inhibition at transcriptional level of IGF-2 gene.

Interestingly, the different expression and distribution of active subunits for type I IFN receptor (IFNAR-1 and IFNAR2-c) could predict, at least in pancreatic cancer cells (**chapter III**), the sensitivity to IFN treatment. In fact, at immunocytochemistry the staining for both subunits was high and mainly membranous in BxPC-3, the most sensitive cell line. While, Panc-1, the most resistant cell to IFN, showed a lower and preferential cytoplasmatic positivity for IFNAR-1, and about 60-70% of the cells were negative for IFNAR-2c. We need to confirm these data with a larger series of pancreatic cancer cell lines and primary cultures, but it is possible that the intensity, the subcellular localization and distribution of IFNs receptor could predict the response to type I IFNs treatment. In the case that this hypothesis will be confirmed, from a practical perspective, a selection of patients to treat with IFN- β could be decided on the basis of IFNAR-1 and IFNAR-2c positivity and localization in the tumor.

The less potent effect of IFN- α compared to IFN- β could be related to the lower affinity for the relative receptor, but it is also possible that human cells have developed resistance mechanisms to avoid being killed by IFN- α , probably upregulating specific survival signalling pathways. In fact, IFN- α induces different survival pathways (fig.1) that protect tumour cells from different stress-producing events (i.e. viral infections). One of these survival pathways is mediated by phosphatidylinositol 3 kinase (PI3K) and by several

of its downstream targets (21). In detail, after IFN- α binding, the IFNAR-1 subunit recruits and activates STAT-3 protein. The activated STAT-3 acts as adapter for PI3K, forming a final complex composed of IFNAR-1, STAT-3, p85 and PI3K (22). One of the targets downstream to PI3K is the serine-threonine kinase Akt, which is important for the generation of antiapoptotic signals such as the phosphorylation, and consequent inactivation, of caspase-9, Bad, and Forkhead transcription factor FKHRL1 (which modulates the Fas-ligand gene expression). Another effect induced by IFN- α is the induction of the ubiquitin cross-reactive protein (ISG 15) and of two ubiquitin-conjugating enzymes (UbcH5 and UbcH8). A molecular target of these enzymes is the inhibitor of κ B (I κ B) protein, a cytoplasmic protein that binds and inactivates NF- κ B. The family of NF- κ B transcription factors regulates the expression of a wide spectrum of genes involved in immunity, inflammation, and cell growth, including apoptosis. Therefore, I κ B degradation activates Rel/NF- κ B that triggers the transcription of genes involved in the antiapoptotic process. In parallel there is another survival pathway, activated by type I IFNs, called “noncanonical”, which involves the linkage of TNF receptor-associated factors (TRAFs) and NF- κ B-inducing kinase (NIK) (23,24).

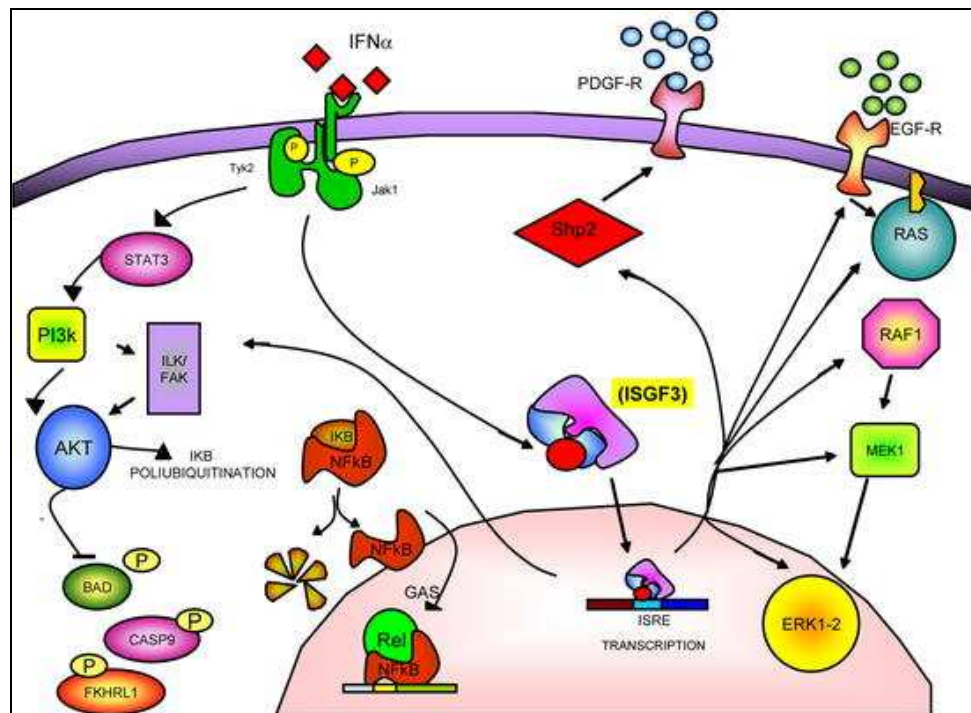


Fig. 1 Escape mechanisms to the antitumour effects of IFN- α . Survival and antiapoptotic signalling are induced by IFN- α through the activation of different pathways. In detail, the following have been reported: the hyperactivation of the EGF-mediated Ras/Raf/Erk-1/2-dependent pathway; the induction of the Akt and NF κ B-dependent pathways through the activation of the STAT3/PI3 K-mediated signalling; and the stimulation of Shp2 that determines an increased response to peptide growth factors

You et al. (25) have studied other targets involved in the IFN- α -activated pathway, such as the Shp1 and Shp2 proteins. After IFN- α binding, Shp1 is associated to the IFN- α receptor, complexed to Jak1 and Tyk2. In this complex, it exercises a negative feedback by inhibiting the IFN- α -stimulated Jak/STAT pathways. Moreover, Shp2 has a positive effect on the mitogenic stimulation of Erk-1/2 and on the expression of platelet-derived growth factor receptor and, on the other hand, inhibits JNK under cellular stress (25). Other Jak/STAT pathway inhibitors have been recently identified, such as the suppressors of cytokine signalling (SOCS)-1 and 2 (26). These two proteins contain an SH2-domain and can inhibit Jak, thus playing a negative control on the Jak/STAT signalling. Other antiapoptotic factors are the protein inhibitors of activated STAT (PIAS) proteins. In detail, PIAS1 (27) inhibits the STAT-1 DNA binding activity and the consequent induction of gene expression. Moreover, Ki-Ras can inhibit the expression of IFN- α -regulated genes providing a novel mechanism of tumour cell escape to the cytokine (28).

In the **chapter V**, we showed another important survival pathway Epidermal Growth Factor (EGF)-dependent that IFN- α regulates in human oropharyngeal epidermoid carcinoma KB cell line. IFN- α is able to increase EGF-receptor expression and function and to activate its downstream targets that include the Ras/MEK1/Erk-1/2 pathway. This effect appears to protect the cells from apoptosis. Selective inhibition of this pathway at different molecular levels always results in increased apoptosis in cells exposed to IFN- α . In fact, either the transfection of a dominant form of Ras RASN17 or the treatment of tumour cells with a specific MEK-1 inhibitor (PD098059) strongly potentiated the apoptosis induced by the cytokine, suggesting that the integrity of Ras function is necessary to produce an anti-apoptotic signal that mediates a survival response in cells exposed to IFN- α via Erk-1 and -2 activation. An additional important finding is that PD098059 specifically abrogated the recovery from apoptosis induced by EGF in IFN- α -treated cells. Therefore, our results suggest that the activation of Ras->Raf-1->Mek1->Erk-1/2 signalling has a prominent role in the anti-apoptotic effects exerted by EGF in epidermoid cancer cells exposed to IFN- α providing evidence of the potential benefits of the molecular interference with this pathway.

In conclusion, the devastating effects of IFN- β on cancer cells support the clinical attractiveness to use this cytokine in the treatment of endocrine and non-endocrine pancreatic tumors and adrenocortical carcinomas. In addition, tumors where the effect of type I IFNs is counteracted by escape mechanisms consisting in increase EGF-receptor expression and function, the activity of IFN could be potentiated by means of highly selective agents like the EGF-

receptor inhibitor Gefitinib (IRESSA; Astra Zeneca, UK) or the dominant negative RASN17 (transfected with recombinant adenovirus). Future studies are necessary to evaluate *in vivo* these promising tools.

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Curriculum Vitae

The author of this thesis was born on the 14th of August 1971 in Naples – Italy. After his graduation at high school (Liceo Scientifico) with maximal score (60/60), he attended the Medical School at the University Federico II of Naples, where he graduated cum laude and special “Academic Mention” for the curriculum in 1995. In 1996 he obtained the license to practice as a Medical Doctor in Naples with maximum mark (90/90). Between 1996 and 2001, the author attended the School of Specialization in Endocrinology and Metabolic Diseases (Chair: Prof. Gaetano Lombardi) - University “Federico II” of Naples. In 2001, he obtained the Specialization in Endocrinology and Metabolic Diseases cum laude. Since October 2003 he is research fellow at Department of Internal Medicine of the Erasmus University (Erasmus Medical Center) of Rotterdam, The Netherlands.

He is author of about 50 publications in peer reviewed journals.

Honours and Awards

- Best poster at the congress of “Giornate Scientifiche della Facoltà di Medicina e Chirurgia e della Facoltà di Farmacia”, Naples (May 1996).
- Under 30yrs award “Cassano Prize - Endocrinological Italian Society” in recognition of the results achieved in Endocrinological Research regarding Neuroendocrine Tumours (September 2001).
- Scholarship from the Huygens Programme (Netherlands Organization for International Cooperation in Higher Education). Programme: “Clinical and Experimental Neuroendocrinology” (2003).
- UICC Yamagiwa-Yoshida Memorial International Cancer Study Grant, from “International Union Against Cancer”.
- Best Presentation for the Oral Communication: “Vitale G, van Eijck CH, van Koetsveld PM, Speel EJ, Colao A, van der Wansem K, Erdmann JI, Lamberts SWJ, Hofland LJ. Potent inhibition of human pancreatic cancer cell growth by beta-interferon”, 3rd BIC Conference – Pancreatic Cancer 2004. Schloss Biebrich Wiesbaden, Germany, 8-9 Ottobre 2004.
- Travel Grant for the Abstract: “Vitale G, van Eijck CH, van Koetsveld PM, Speel EJ, Colao A, Lombardi G, van der Wansem K, Erdmann JI, Lamberts SWJ, Hofland LJ. Potent inhibition of human pancreatic cancer cell growth by beta-interferon. Endocrine Society Meeting, San Diego 4-7/7/05.

Selection of Publications (2001-2006)

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